Role of PI3Kβ and PI3Kγ Isoforms in Sphingosine 1-Phosphate (S1P)-Induced Endothelial Cell Migration

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Content

1. Introduction

1.1 Phosphoinositide 3-kinases (PI3Ks)

PI3Ks comprise a family of kinases that catalyse the transfer of the γ-phosphate group of ATP to the D3 position of phosphoinositides to generate $PI_{(3)}P$, $PI_{(3,4)}P_2$, and $PI_{(3,4,5)}P_3$. This process can be repealed by the phosphatase and tensin homolog (PTEN) tumour suppressor protein or by Src-homology 2 (SH2) domain-containing inositol 5-phosphatase (SHIP) which dephosphorylate at the 3´-OH and 5´-OH position of the inositol ring, respectively. Since the discovery of a PI3K activity in 1988, eight PI3K catalytic subunits have been identified in mammals. Based on their different structure, substrate specificity and regulatory mechanisms, PI3Ks are divided into three main classes (Domin and Waterfield, 1997).

1.1.1 Class I PI3Ks

Class I PI3Ks are present in most cell types as heterodimeric proteins, consisting of a 110-120 kDa catalytic subunit and an associated regulatory subunit. *In vitro*, they are able to phosphorylate PI, $PI_{(4)}P$, and $PI_{(4,5)}P_2$, whereas *in vivo* their preferred substrate is $PI_{(4,5)}P_2$. Based on structural and functional differences, class I PI3Ks can be further divided into two subclasses, which signal downstream of receptor tyrosine kinase (RTK) and heterotrimeric G protein-coupled receptors (GPCR), respectively.

Class IA PI3K

Three isoforms of class IA PI3K have been found in mammals. They were cloned and designated PI3Kα (Hiles et al., 1992), PI3Kβ (Hu et al., 1993) and PI3Kδ (Vanhaesebroeck et al., 1997). PI3Kα and PI3Kβ are widely expressed in mammalian

tissues, while PI3Kδ is mainly expressed in leukocytes. All members are tightly and constitutively associated with a 50-85 kDa regulatory subunit. Based on the molecular weight, they have been named p85α, p85β, p55α, p55γ, or p50α (Katso et al., 2001). The catalytic subunit of class IA PI3K consists of four regions (Fig. 1): the N-terminal p85-binding domain, which binds to the regulatory subunit; the Ras-binding domain, which is unique to class I PI3K; the phosphoinositide kinase homology domain (PIK), which is conserved in all lipid kinases, and a C-terminal catalytic domain.

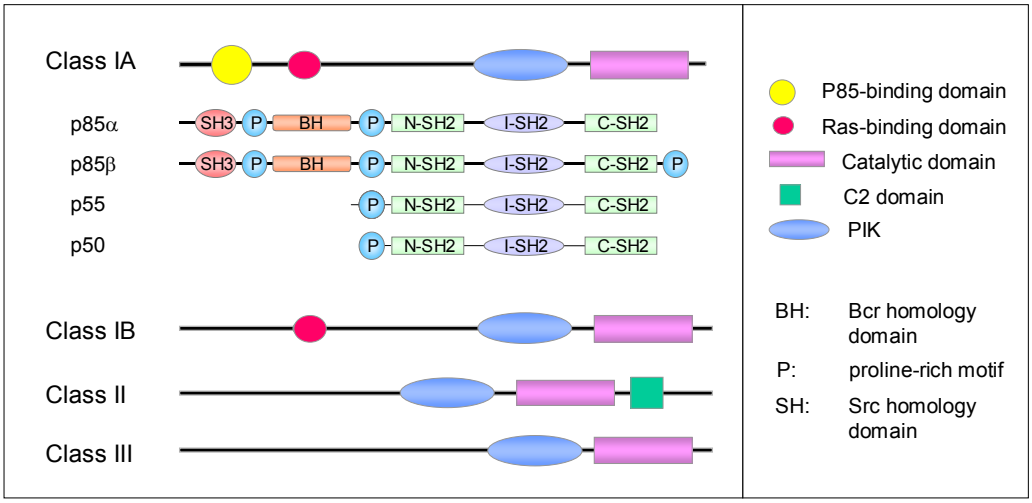

Figure 1. Schematic diagram of catalytic and regulatory subunits of the heterodimeric phosphoinositide 3-kinases.

The regulatory subunits do not possess any known enzymatic activity but are composed of several domains with homology to those found in other signalling proteins (Fig. 1). p85α and p85β contain an N-terminal Src-homology 3 (SH3) domain, a Bcr homology (BH) domain surrounded by two proline rich regions, and three SH2 domains. The p50 and p55 adaptor proteins share the C-terminus of p85 and have a unique N-terminal sequence that lacks an SH3 motif, one proline rich region and the BH domain.

In response to many cellular stimuli, the SH2 domains of the PI3K regulatory subunits bind selectively to the phosphorylated tyrosine residues of RTKs leading to the

translocation of the p110 catalytic subunit from the cytosol to the plasma membrane. This recruitment of p110 subunits is believed to be vital for PI3K activation. At the plasma membrane, activated class IA PI3Ks can readily access their lipid substrates, leading to a local increase in the production of $PI_{(3,4,5)}P_3$ which acts as a critical messenger in the regulation of several signalling pathways and different cellular functions, such as cell survival, proliferation and differentiation. In addition to lipid kinase activity, class IA PI3Ks exhibit an intrinsic protein kinase activity. The major substrates are serine residues within the catalytic subunit itself or its associated regulatory subunit. For instance, p110α was able to phosphorylate its regulatory subunit p85α at serine 608 (Yu et al., 1998). In contrast, p110β and p110δ did not phosphorylate their adaptor molecules but express the capability to autophosphorylate serine 1070 of p110β (Czupalla et al., 2003) or serine 1039 of p110δ (Vanhaesebroeck et al., 1999a), respectively. Interestingly, the p110α-mediated p85 phosphorylation or p110δ autophosphorylation led to diminished lipid kinase activity of the enzymes, while autophosphorylation of p110β did not alter its lipid kinase activity (Czupalla et al., 2003; Vanhaesebroeck et al., 1999a).

Additionally, it should be mentioned that not only tyrosine kinases, but also Gβγ heterodimers express the ability to activate p110β. It has been shown that the activity of p85/p110β was stimulated by G protein βγ subunits in cell-free systems (Hazeki et al. 1998). Moreover, Murga et al. have shown that p110β was required to stimulate Akt through a GPCR pathway in murine fibroblasts *in vivo* (Murga et al., 2000). Similarly, in bovine aortic endothelial cells (BAEC), sphingosine 1-phosphate (S1P) stimulation resulted in a rapid and isoform-specific activation of p110β in a Gβγ-dependent manner (Igarashi and Michel, 2001b)

Class IB PI3K

The unique member of class IB PI3K identified to date is the p110γ catalytic subunit with 36 % identity to p110α. The first indication for this isoform were observed in

neutrophils stimulated with a formulated tripeptide, formyl-methionine-leucine-phenylalanine (fMLP), which led to a rapid increase in $PI_{(3,4,5)}P_3$ levels (Stephens et al., 1993). Screening a human bone marrow cDNA library, Stoyanov et al. subsequently cloned and characterised a novel PI3K isoform, PI3Kγ, which associates with and is activated by G_i protein βγ subunits (Stoyanov, 1995). PI3Kγ was initially believed to be mainly expressed in haematopoietic cells. However, recent studies provided evidence for a wider expression pattern of PI3Kγ in other tissues like smooth muscle, kidney and heart. The broad distribution of PI3Kγ illustrates its importance in the regulation of diverse cell functions.

The three dimensional structure of PI3Kγ has been determined (Walker et al., 1999) (Fig. 1). The catalytic subunit is composed of a C-terminal catalytic domain, a C2 domain, a PIK domain, a Ras binding domain (RBD), and an N-terminal region, which shares some similarity to the Pleckstrin homology (PH) domain of Rho-GAP (GTPase-activating proteins) and was previously proposed to be important for the interaction with the regulatory subunit (Krugmann et al., 1997). Different from members of PI3K class IA, PI3Kγ was found in association with a novel 101 kDa adaptor protein, p101 (Stephens et al., 1997). In addition, a new adaptor protein of PI3Kγ, $p87^{PIKAP(PI3K\gamma\ adaptor\ protein)}$ (p87, also termed p84), has been identified with a homologous region to p101 within the p110γ and Gβγ-interacting domains (Voigt et al., 2006; Suiro et al., 2005). Its gene locates next to the p101 locus and exists in many mammals. In a monkey SV40-transformed kidney fibroblast cell line (COS-7) and a human embryonic kidney epithelial cell line (HEK293), p101 is predominantly distributed within the nucleus in the absence of p110γ, and translocated to the cytosol when p110γ is coexpressed. On the contrary, p87 is present in the cytosol regardless of the existence of p110γ (Voigt et al., 2006). Both adaptors bind to the p110γ catalytic subunit with a comparable affinity (Surie et al., 2005) whereas the affinity of Gβγ binding seems to be higher for p101 compared to p87 (Surie et al. 2005). However, the association of p87 with Gβγ could not be detected by another group (Voigt et al., 2006).

Despite being termed adaptor/regulatory subunits, the functional relevance of the p101 and p87 to date is limited. It has been demonstrated that coexpression of p110γ/p101 in the insect cell line Sf9 and in COS-7 cells resulted in a significant increase in $PI_{(3,4)}P_2$ and $PI_{(3,4,5)}P_3$ production as compared to the expression of p110γ alone. In agreement, fMLP-induced increase of $PI_{(3,4,5)}P_3$ generation and PI3Kγ activation were dependent on the expression of either p101 or p87 (Stephens et al., 1997; Voigt et al., 2006). Additional finding proposed that p101 is responsible for the $PI_{(4,5)}P_2$ substrate selectivity of p110γ, by sensitizing p110γ towards Gβγ in the presence of $PI_{(4,5)}P_2$ (Maier et al., 1999). However, several reports demonstrated that p110γ can associate with Gβγ directly and that p110γ lipid kinase activity was substantially activated by Gβγ even in the absence of p101 subunit (Stoyanov et al., 1995; Tang and Downes, 1997; Leopoldt et al., 1998), arguing against an indispensable role of p101 in Gβγ stimulation of p110γ.

The PI3Kγ activation can be induced by many agonists through GPCR, such as monocyte chemoattractant protein (MCP-1), stromal-cell derived factor 1 (SDF-1), fMLP, and interleukin-8 (IL-8). In response to stimulation, PI3Kγ displays not only lipid kinase activity, but also protein kinase activity. Utilizing PI3Kγ mutants with aborted lipid kinase activity, Bondeva et al. demonstrated that MAPK was activated in the complete absence of $PI_{(3,4)}P_2$ and $PI_{(3,4,5)}P_3$ production (Bondeva et al., 1998). Based on these findings, the authors suggested that PI3Kγ is capable of activating two distinct pathways: the lipid kinase activity of PI3Kγ is responsible for 3-phosphorylated lipid production while the PI3Kγ protein kinase activity contributes to MAPK stimulation and propagation of growth signals. Moreover, recombinant PI3Kγ has been indicated to catalyse transphosphorylation of the adapter protein p101 and the protein kinase MEK-1, which was completely abolished by the PI3K inhibitor wortmannin (Bondev et al., 1999). Czupalla et al. showed that PI3Kγ was also able to autophosphorylate the p110γ catalytic subunit at serine 1101 residue, which was also stimulated by Gβγ subunits in a time- and concentration-dependent manner. Interestingly, the autophosphorylation of PI3Kγ had no inhibitory effect on its lipid kinase activity (Czupalla et al.,

2003).

Due to the relatively recent discovery of PI3Kγ, the functional significance of PI3Kγ is only preliminarily determined. To investigate the role of PI3Kγ in biological responses, mice lacking p110γ have been generated. The absence of PI3Kγ in mice led to an increased population of neutrophils, monocytes and eosinophils. In contrast, the proliferation and activation of thymocytes were reduced (Sasaki et al., 2000). In response to chemoattractants such as fMLP, complement factor 5a (C5a) and IL-8, the production of $PI_{(3,4,5)}P_3$ as well as the phosphorylation of ERK and Akt were abolished in PI3Kγ$^{-/-}$ neutrophils. Accordingly, fMLP- and C5a-stimulated neutrophil migration and respiratory burst were remarkably reduced in cells lacking p110γ (Sasaki et al., 2000; Li et al., 2000; Hirsch et al., 2000; Hannigan et al., 2002). The role of PI3Kγ has also been investigated in bone marrow-derived mast cells (BMMC). Mast cells are key players in allergy and inflammation. In PI3Kγ-deficient BMMCs, $PI_{(3,4,5)}P_3$ production as well as $PI_{(3,4,5)}P_3$-dependent Akt phosphorylation were impaired in response to adenosine, a GPCR agonist, whereas no alteration was observed in both events upon challenge with agonists coupled to RTK (e.g. IL-3, stem cell factor-1). These results confirm that p110γ is activated by G-protein coupled receptors but not by RTKs.

To date, the investigations of the role of PI3Kγ were mainly performed in haematopoietic cells. Although the expression of this protein has also been observed in endothelial cells very recently, the function of PI3Kγ in this cell type is poorly characterised.

1.1.2 Class II and III PI3Ks

Members of class II PI3Ks are approximately 170-210 kDa, containing three groups, PI3K II α, β and γ. They are composed of a C-terminal C2 domain, which has been implicated to bind to phospholipids *in vitro* (MacDougall et al. 1995) and an N-terminal region that contains a Ras binding domain. *In vitro*, Class II PI3K can be activated by

growth factors, integrins and chemokines. Upon activation, these enzymes phosphor-rylate PI and $PI_{(4)}P$ to produce $PI_{(3)}P$ and $PI_{(3,4)}P_2$ with a strong preference for PI.

A well-known class III PI3Ks isoform is the Saccharomyces cerevisiae VPS34 (vesicular-protein-sorting) gene product, Vps34p (Schu et al., 1993). It has been shown in association with a Vps15p protein serine/threonine kinase in yeast (p150 in mammals), which is essential for the recruitment of Vps34p to the Golgi membranes and activation of its lipid kinase activity. *In vitro*, class III PI3Ks utilise PI as a substrate and are likely to be responsible for the production of most of the $PI_{(3)}P$ in cells.

1.2 Signalling reactions of PI3-kinases

A number of proteins have been identified that directly bind to $PI_{(3,4)}P_2$ and $PI_{(3,4,5)}P_3$ via their PH domain, such as protein kinases (Akt/protein kinase B (PKB), phosphoinositide-dependent protein kinase 1 (PDK1) and Bruton's tyrosine kinase (Btk)), guanine nucleotide exchange factors (GEFs) such as Vav and Sos1, GTPase-activating factors (GAP1), phospholipases (PLCγ2, PLCδ1), and adaptor proteins.

1.2.1 Akt/PKB

Akt, also termed PKB, is one of the best characterised downstream effectors of PI3Ks. It was cloned in 1991 as a cellular homologue of the retroviral oncogene v-akt (Bellacosa et al., 1991; Coffer & Woodgett, 1991; Jones et al., 1991), and consists of an N-terminal PH domain, a kinase domain and a C-terminal regulatory region. The binding of the PH domain to $PI_{(3,4,5)}P_3$ recruits Akt from the cytosol to the plasma membrane, where it can be activated via phosphorylation of threonine 308 and serine 473 (Alessi et al., 1996).

Activated Akt mediates several biological events via different downstream substrates. For instance, Akt was able to directly phosphorylate mTOR/FRAP, then leading to the phosphorylation of eIF-4E binding protein (4E-BP) and activating mRNA translation (Gingeras et al., 1999). Furthermore, Akt is responsible for regulation of cell apoptosis. p70 S6 kinase ($p70^{S6K}$), an anti-apoptotic effector, has been demonstrated to be directly activated by Akt (Zhang et al., 2005) and negatively regulates BAD (Bcl-xL/Bcl-2-associated death promoter)-induced apoptosis (Harada et al., 2001). Moreover, BAD is also a downstream target of Akt, which is directedly phosphorylated by Akt and thus, inhibites cell apoptosis by releasing the anti-apoptotic proteins Bcl-X_L and Bcl-2 (Datta et al., 1997). Alternatively, Akt has also been known as a promoter of cell survival. Direct phosphorylation of glycogen synthesis kinase-3 (GSK-3) by Akt led to a decrease in GSK-3 activity inhibiting glycogen synthesis and mediating endothelial cell survival (Kandel and Hay, 1999; Kim et al., 2002; Skurk et al., 2005). Important stimuli of Akt, including vascular endothelial growth factor (VEGF) and S1P, activate Akt through PI3K, thereby promoting cell survival (Alon et al., 1995; Igarashi et al., 2001a).

Former studies have revealed a role of Akt in cell migration. Interestingly, more recent studies suggest that Akt may play both negative and positive roles in the regulation of cell migration in an isoform-specific manner. Irie et al. have shown that Akt1 downregulated migration in epithelial cells (Irie et al., 2005) and breast cancer cells while Akt2 functioned as a positive regulator (Yoeli-Lerner et al., 2005; Wyszomierski et al., 2005). In contrast, in response to PDGF, the Akt1 deficiency reduced migration of mouse embryo fibroblasts, whereas Akt2 deficiency increased the cell movement by increasing the activities of the small GTPase Rac and p21 activated kinase 1 (PAK1) (Zhou et al., 2006). Similarly, Akt positively regulates migration of endothelial cells which predominantly express Akt1 (Chen et al., 2005). In these cells, Akt activation and the subsequent migration were not only induced by various growth factors, but also by bioactive lysophospholipids, in particular by S1P (see below). One downstream target involved in this process is endothelial nitric oxide synthase (eNOS)

which is phosphorylated by Akt and seems to be required for directional cell movement in response to VEGF (Morales-Ruiz et al., 2001). Coordinately, Ackah et al. has indicated that Akt1 is importance for endothelial cell migration via stimulating nitric oxide (NO) production (Ackah et al., 2005). Additionally, important downstream substrates of Akt in the regulation of cell migration appear to be Rho family GTPases, although several groups have suggested that the small GTPase Rac may also be upstream of Akt phosphorylation (Lee et al., 2001; Gonzalez et al., 2006). However, the mechanism of this process seems to be complex and needs more clarification.

1.2.2 Rho GTPases

The Rho GTPase family belongs to the Ras superfamily of monomeric 20-30 kDa GTP-binding proteins, sharing approximately 25 % sequence identity to Ras. To date, 21 members have been discovered in mammals. Based on their primary sequence and function, they can be divided into five subgroups: Rho-like, Rac-like, Cdc42-like, Rnd and RhoBTB (Burridge et al., 2004). These proteins cycle between inactive GDP-bound state and active GTP-bound state. In the active state, they are able to interact with downstream effectors and transmit signals. Binding of targets terminates responses by hydrolysis of the protein-bound GTP. The interconversion between inactive and active states is regulated by three types of proteins: GTPase-activating proteins (GAP), which stimulate intrinsic GTPase activity; guanine nucleotide dissociation inhibitors (GDI), which inhibit the exchange of GDP for GTP, and guanine nucleotide exchange factors (GEF), which catalyse the exchange of GDP for GTP and therefore induce the accumulation of the active GTP-bound form. Several GEFs are known to be downstream mediators of Class I PI3Ks.

Three members, Rho, Rac, and Cdc42, have been well characterised during the last few years. They have been shown to contribute to the regulation of several cell responses such as enzyme activation, cell polarity, gene transcription, cell cycle progression and survival (Hall, 2005). Moreover, they were characterised as key

regulators being responsible for the rearrangement of actin cytoskeleton and the promotion of cell motility by different mechanisms (Fig. 2). Rho was shown to regulate stress fibers formation, focal adhesion and cell contraction, whereas Rac and Cdc42 stimulate lamellipodia/ruffles production and filopodia formation, respectively (Ridley and Hall, 1992; Nobes and Hall, 1995).

Although Rho and Rac have been shown to mediate cell migration in a coordinated manner, more recent studies focused on the influence of Rac in promoting cell chemotaxis. Activation of Rac1 was found to be necessary for cell migration in different cell types including glioma cells, neutrophils, smooth muscle and endothelial cells (Malchinkhuu et al., 2005; Pestonjamasp et al., 2006; Goueffic et al., 2006). Several studies have demonstrated that Rac activation occurs via pathways involving PI3K and/or Akt. For instance, chemokine-stimulated Rac activation via a G_i protein/PI3K pathway was critical for macrophage migration (Weiss-Haljiti et al., 2004) and in embryo fibroblasts active Rac1 mediates cell migration in an Akt-dependent manner (Zhou et al., 2006; Qian et al., 2004). In endothelial cells, S1P stimulated Rac in a $S1P_1$/PI3K/Akt-dependent manner, involving the phosphorylation of $S1P_1$ by the Akt activation (Lee et al., 2001). Furthermore, the migration was increased in cells coexpressing a GEF, P-Rex2b, with $G\beta\gamma$ and/or PI3K (Li et al., 2005). Recently, it became apparent that a downstream effector of Rac, cortactin, was involved in Rac-dependent cytoskeleton remodeling. Upon the cell stimulation, cortactin translocates to actin polymerisation sites on plasma membrane, thus regulating actin rearrangement and cell migration. This response was inhibited by expression of a dominant negative Rac mutant in the cells (Vouret-Craviari et al., 2002). The interaction of Cortactin with Arp2/3 (Lee et al., 2006a) or with zonula occludens-1, a tight junction associated protein (Lee et al., 2006b) was involved in this process.

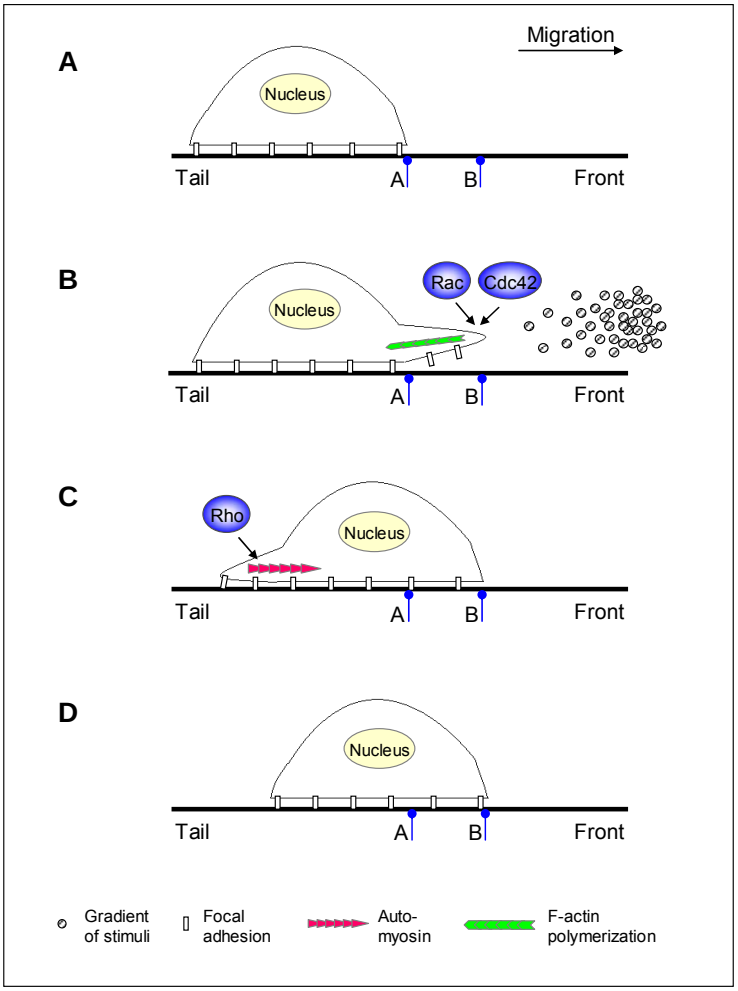

Figure 2. Distinct roles of Rho family small GTPases in regulating cell migration (from position A to B). In response to a chemoattractant gradient, migrating cell extends lamellipodia/filopodia at the leading edge. The response is dependent on the activation of Rac/Cdc42, which subsequently promotes localised actin polymerisation. Furthermore, the cell body follows the front of the cell and the trailing edge retracts via enhanced actomyosin contractility which is manifested by Rho-dependent stress fiber formation. (Adapted from Fenteany and Zhu, 2003)

Taken together, these findings indicate the essential role of PI3K/Rac signalling pathway in the regulation of cell motility. However, the mechanism of PI3K-regulated Rac activity and the involved signalling molecules are not well characterised.

1.3 Cell migration and endothelium

The endothelium is the monolayer of endothelial cells lining the lumen of blood vessels. The structure and functional integrity of endothelial cell are important in the maintenance of the vessel wall and circulatory function. Moreover, the endothelium is responsible for the regulation of vascular tone, blood flow, metabolic, synthetic, anti-inflammatory and antithromobogenic processes as well as for angiogenic vascular remodeling (Galley et al., 2004), and is indispensable for body homeostasis. Accordingly, an uncontrolled endothelial response is involved in many disease processes, including atherosclerosis, hypertension and inflammatory syndromes.

A major function of endothelial cells is the regulation of angiogenesis, which is defined as the process by which new blood vessels are formed from the pre-existing vasculature. Under physiological conditions, angiogenesis is tightly regulated and plays an essential role in development, reproduction, inflammation and wound healing. However, unregulated angiogenesis can occur under pathological conditions and contributes to numerous diseases, including tumour growth, diabetic retinopathy and rheumatoid arthritis. Angiogenesis involves several major processes such as increased permeability, degradation of the extracellular matrix by proteolytic enzymes, migration and proliferation of endothelial cells, and morphological differentiation into three dimensional tubular structures (Carmeliet et al., 2003). Thus, to achieve new blood vessel formation, endothelial cells escape from their stable location by breaking through the basement membrane and migrate toward an angiogenic stimulus released from adjacent tumour cells, activated blood cells, or wound-associated macrophages. Behind this migrating front, endothelial cells proliferate and finally, the new outgrowth of endothelial cells reorganises into capillaries which are stabilised by the formation of a new basement membrane and the recruitment of pericytes. Dysregulation of angiogenesis can result in insufficient vascularisation or in the formation of immature vessels (Carmeliet et al., 2003). On the other hand, uncontrolled angiogenesis supports tumour progression. Angiogenesis is controlled

by a balance between stimulatory and inhibitory factors. Growth factors, in particular VEGF, are essential for most steps in angiogenesis. Recently, however, other mediators such as S1P that accumulates at high levels at sites of vascular injury have been observed to potently contribute to angiogenesis (see below) (Ancellin et al., 2002).

In order to target unwarranted angiogenesis or to be able to stimulate angiogenesis in ischemic tissues, the underlying mechanisms including signalling pathways regulating cell migration need to be fully clarified. Endothelial cell migration is a critical event in angiogenesis which is triggered by several agonists including VEGF, fibroblast growth factor (FGF), integrins and as discussed below, S1P. Cell migration involves sensing of a chemokine, organisation of signalling asymmetry, cytoskeleton reorganisation with localised actin polymerisation at the leading edge, new cell adhesion at the front of the cell and contraction of the body and rear of the cell. The signalling molecules regulating endothelial migration have, at least in part, been introduced in the preceeding paragraphs and involve PI3Ks, Akt, eNOS and Rac1. Importantly, although the role of PI3K isoforms in neutrophil migration has been intensively investigated, their function in endothelial migration is hardly known. For instance, polarised PI3Kγ activity and $PI_{(3,4,5)}P_3$ production are thought to organise the leading edge of leukocytes by, at least in part, asymmetrical recruitment of Rho GTPases which are involved in actin reorganisation (Rickert et al. 2000). Similar processes may occur in endothelial cells and the contribution of different PI3K isoforms may be stimulus-dependent and may involve synergistic as well as specific reactions. The clarification of these signalling pathways will lead to a better understanding of endothelial migration and finally, angiogenesis.

1.4 Sphingosine 1-Phosphate (S1P)

S1P is a polar lysophospholipid metabolite that has been proposed to act as an extracellular mediator and an intracellular second messenger. Platelets which are rich

source of S1P release it upon activation by prothrombotic stimuli such as thrombin, ADP, and collagen. S1P is also synthesised in a wide variety of other cell types in response to extracellular stimuli such as growth factors and cytokines. The concentration of S1P in serum is about 0.5 µM, which is considerably higher than in plasma (0.1 µM). The biosynthesis of S1P has been well characterised and starts with the breakdown of a number of membrane phospholipids to produce sphingomyelin that is cleaved by sphingomyelinase and ceramidase to generate sphingosine (Fig. 3). Furthermore, sphingosine kinase catalyzes the phosphorylation of sphingosine to sphingosine 1-phosphate. Conversely, S1P can either be degraded via dephosphorylation by S1P phosphatase back to sphingosine or cleaved by S1P lyase to produce hexadecanal and phosphoethanolamine.

S1P regulates various biological functions in many vertebrate cell types, including proliferation, migration, apoptosis, cytoskeletal organisation and differentiation. Therefore, S1P has been implicated in many physiological processes and pathophysiological states, such as wound healing, inflammation, cancer and tumour angiogenesis. Most effects are mediated via signalling pathways activated by binding of S1P to its receptors.

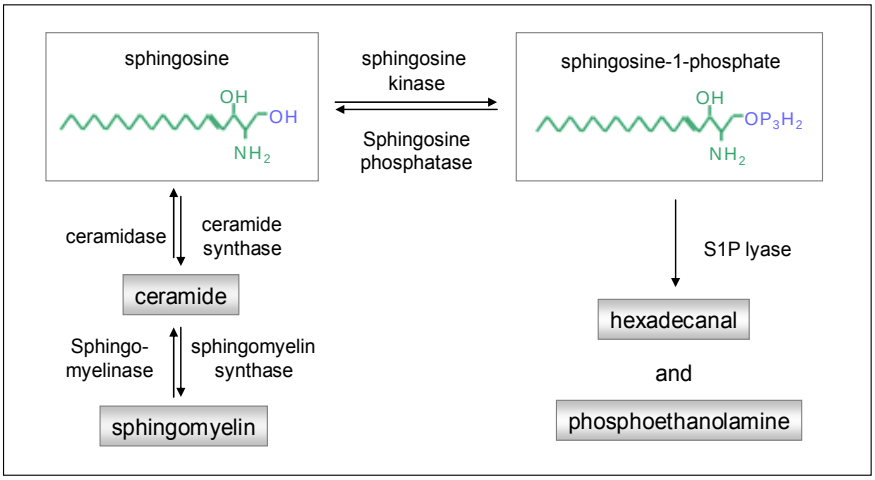

Figure 3. S1P synthesis and degradation
(Adapted from Alewijnse et al. 2004)

1.4.1 S1P receptors

The receptors of S1P were cloned and characterised as a family of GPCR, previously known as endothelial differentiation genes (Edg), which can be divided into two subgroups based on ligand specificity. One group exhibits high affinity for lysophosphatidic acid (LPA) and consists of three members: Edg-2, Edg-4, Edg-7 (also known as LPA-1, LPA-2, and LPA-3). The second subgroup comprises five isoforms which display high affinity for S1P: Edg-1, Edg-3, Edg-5, Edg-6 and Edg-8, which have been recently renamed as $S1P_1$, $S1P_3$, $S1P_2$, $S1P_4$, and $S1P_5$, respectively.

$S1P_1$ (Edg-1)

$S1P_1$, the first member of S1P receptors, was originally cloned and characterised as an immediate-early gene product in phorbol ester-differentiated human umbilical vein endothelial cells (HUVEC) (Hla et.al., 1990). It is a protein composed of 380 amino acids with seven hydrophobic, transmembrane spanning domains and it binds to S1P with a high affinity (K_d 8-20 nM). Following S1P stimulation, $S1P_1$ associates with the pertussis toxin (PTX)-sensitive heterotrimeric $G_{i/O}$-protein with high affinity. Additionally, Lee et al. suggested that $S1P_1$ phosphorylation catalysed by its downstream effector Akt is indispensable for S1P-induced signalling pathways (Lee MJ. et al., 1999).

$S1P_1$ is widely expressed in different tissues and cell types, such as immune, neuronal, smooth muscle and endothelial cells, suggesting its importance in regulating different physiological and pathological responses. In endothelial cells, many cellular effects of S1P have been proposed to be mediated by $S1P_1$ activation.

$S1P_3$ (Edg-3)

Expression of $S1P_3$ is also detected in endothelial cells. It was initially cloned from a human genomic library during a search for human cannabinoid receptors. Different from $S1P_1$, it is expressed in HUVEC at lower level (Wang et al., 1999a) and interacts not only with heterotrimeric G_i proteins, but also with G_q, G_{12} and G_{13}. $S1P_3$ exhibits a wide tissue expression pattern, with highest levels in endothelial cells, leukocytes and cardiovascular tissue. Surprisingly, deletion of $S1P_3$ in mice results in no visual phenotypic abnormality, indicating that other receptors may be able to compensate for $S1P_3$ function.

1.4.2 S1P-mediated cellular responses in endothelial cells

Recent studies have focused on the molecular mechanisms of receptor-mediated responses to extracellular S1P in endothelial cells. According to these studies S1P is thought to be an important angiogenic mediator regulating different endothelial cell responses. For instance, S1P has been shown to induce proliferation in HUVEC (Lee MJ. et al., 1999; Lee et al., 2000). This response was inhibited by treatment of cells with a $G_{i/o}$ protein-specific inhibitor, PTX (Kimura et al., 2000), suggesting the involvement of a $S1P_1$/Gi signalling pathway. S1P also promotes endothelial tube formation *in vitro* in a Gi-dependent way (Wang et al., 1999a; Lee OH. et al. 1999). Deletion of $S1P_1$ in mice resulted in embryonic lethality due to the incomplete maturation of blood vessels (Lee PC. et al., 1999; Liu et al., 2000). More recently, Thompson et al. have observed an essential role of $S1P_3$ in Ca^{2+}-dependent morphogenesis of endothelial cells in response to S1P (Thompson et al., 2006).
Importantly, the role of S1P in the regulation of endothelial cell motility has been observed. Generally, S1P has been shown to regulate migration in both negative and positive ways. An inhibited migration by S1P has been shown in human breast cancer cells, myoblasts and vascular smooth muscle cells (VSMC, Wang et al., 1999b;

Becciolini et al., 2006; Ryu et al., 2002), whereas stimulation of migration by S1P has been found in fibroblasts, VSMC (Long et al., 2006; Kluk and Hla, 2001) and in endothelial cells.

Several groups have demonstrated that S1P stimulates migration of endothelial cells in a concentration-dependent manner (Panetti et al., 2000; Kimura et al. 2000; Lee et al., 2000). Therefore, the signalling pathways underlying S1P-induced migration have been partially revealed (Fig. 4). For example, S1P-stimulated migration is diminished in $S1P_1$ null cells (Liu et al., 2000). Accordingly, cells expressing a mutant of $S1P_1$, T236AEDG-1 which is unable to be phosphorylated, show reduced migration indicating the importance of $S1P_1$ in this process (Lee et al., 2001). Moreover, S1P-induced migration was decreased by pretreatment with PTX or with the PI3K inhibitors wortmannin and LY294002 (Kimura et al., 2000; Lee et al., 2000), implicating the involvement of a GPCR/Gi/PI3K pathway in S1P-induced endothelial cell migration. It has also been suggested that S1P-dependent transactivation of VEGF receptors via $S1P_1$ and G_i protein leads to activation of Src, and the adaptor protein CrkII is responsible for both the induction of membrane ruffling and the increase in cell motility. Accordingly, the inhibition of the VEGF receptor abolished S1P-induced migration (Endo et al., 2002). Interestingly, VEGF was also able to enhance $S1P_1$ expression (Igarashi et al., 2003; Hughes et al., 2005). These data represent examples for the crosstalk between $S1P_1$ and RTK signalling pathways.

Akt and small GTPases have been identified as downstream effectors of S1P. Upon stimulation with S1P, PI3Ks lead to the activation of protein kinase Akt which in turn phosphorylates eNOS. Furthermore, Akt also mediates the phosphorylation of $S1P_1$, that seems to be essential for the activation of the small GTPase Rac which is involved in cytoskeleton reorganisation and cell migration (Rishiyuki et al., 2002; lee et al., 2001; Panetti et al., 2000). $S1P_3$ is also able to mediate Rac activation in a G_i protein-dependent manner and in addition, it can induce the activation of another small GTPase, RhoA, which regulates actin-myosin interactions necessary for cell motility (Ohmori et al., 2001; Inoki et al., 2006).

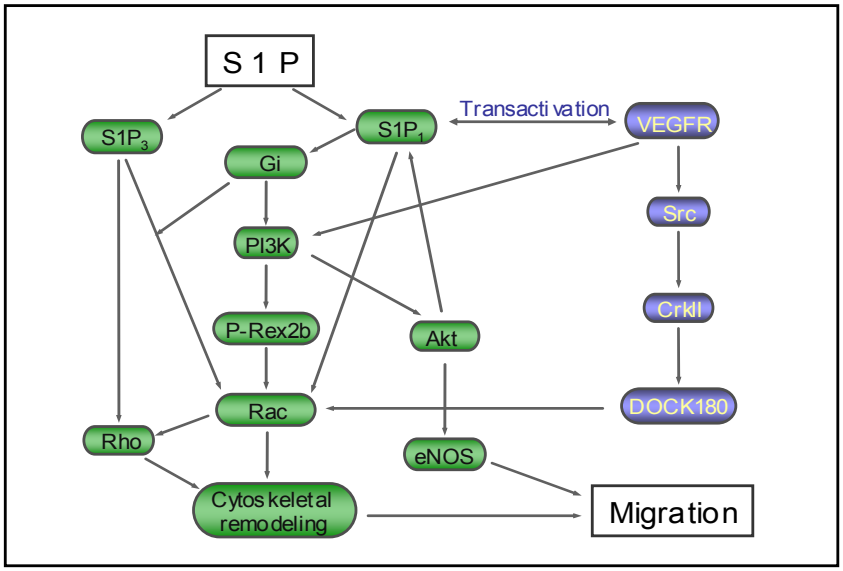

Figure 4. S1P-induced signalling pathways of endothelial cell migration

Taken together, S1P is an important stimulus for many cellular responses, particularly for cell motility. PI3Ks, activated through G_i protein- or tyrosine kinase-dependent signalling pathways, appear to be critical players in S1P-iniated cell migration. Thus, it will be interesting to analyse the regulatory function of PI3Ks in S1P-induced endothelial cell motility.

1.5 Project aim

As described above, PI3Ks, in particular class I, are important enzymes that regulate cell migration in response to different stimuli in neutrophils, monocytes and other cell types. Two isoforms of class I, PI3Kβ and PI3Kγ, have been indicated to be activated by G_i proteins. However, the function of these two isoforms in endothelial cells is not well characterised. Platelet-derived S1P has been demonstrated to mediate PI3K activity via the activation of its Gi protein coupled receptor $S1P_1$ in endothelial cells. It has been demonstrated that this signalling pathway mediates endothelial cell migration which is an essential step in the process of angiogenesis. However, to date, the mechanisms of S1P-induced endothelial cell migration, especially the contribution of PI3Kβ and PI3Kγ, are not fully understood. Moreover, it is not known whether these isoforms act via distinct signalling pathways.

The aim of the present study was to investigate the role of PI3Kβ and/or PI3Kγ in S1P-induced endothelial cells migration. We detected the protein expression of these two isoforms in human and mouse vascular endothelial cells. Their role in regulating the downstream signalling pathways and endothelial cell motility was studied using different PI3Kβ or PI3Kγ isoform-specific tools such as inhibitors, cDNA plasmids and gene knockout murine cells.

2. Materials and Methods

2.1 Materials

2.1.1 Animals

p110$\gamma^{-/-}$ mice, a kind gift from the Dipartimento di Genetica, Biologia e Biochimica, Universita di Torino, Italy, were generated as described before (Hirsch et al., 2000). Both wild-type and p110$\gamma^{-/-}$ animals were bred and housed in the Institute of Laboratory Animal Science of the FSU Jena. About 10-12 week-old littermates from heterozygous crosses were used in the current study.

2.1.2 Primary cells culture

- HUVEC, Human Umbilical Vein Endothelial Cells
- MLEC, Murine Lung Endothelial Cells
  (Preparation see 2.2)

2.1.3 Plasmids

- pcDNA3, mammalian expression vector (Invitrogen, GmbH, Karlsruhe)
- pcDNA3-PI3Kγ KR, kinase dead mutant of PI3Kγ; Lys833 to Arg mutation in catalytic domain
- pcDNA3-PI3Kβ KR, kinase dead mutant of PI3Kβ; Lys805 to Arg mutation in catalytic domain

2.1.4 Antibodies

- Anti-PI3Kγ monoclonal antibody, directed against the N-terminal fragment of PI3Kγ (provided by the Institute of Molecular Cell Biology, University of Jena)

- Anti-PI3Kβ polyclonal antibody (Santa Cruz Biotechnology, Heidelberg)
- Anti-phospho-ERK monoclonal antibody (New England Biolabs, Frankfurt)
- Anti-pan-ERK monoclonal antibody (BD Transduction Laboratories, Heidelberg)
- Anti-phospho-Akt polyclonal antibody, serine 473 (Cell Signalling Technology, Frankfurt)
- Anti-Akt1 monoclonal antibody (Santa Cruz Biotechnology, Heidelberg)
- Anti-phospho-eNOS polyclonal antibody, serine 1177 (Cell Signalling Technology, Frankfurt)
- Anti-eNOS polyclonal antibody (BD Transduction Laboratories, Heidelberg)
- Anti-Rac1 monoclonal antibody (BD Transduction Laboratories, Heidelberg)
- Anti-vinculin monoclonal antibody (Cell Signalling Technology, Frankfurt)
- Peroxidase conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, USA)
- Peroxidase conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, USA)
- Rat anti-mouse CD102 antibody (BD Bioscience, Heidelberg)

2.1.5 Oligonucleotides

Oligonucleotides for human p101-specific RT-PCR

Forward Primer 5'- CTGGGCTGTGTCTGAACTG -3'
Reverse Primer 5'- GTGGTGTAGGCACTGTGAG -3'

Oligonucleotides for murine p101-specific RT-PCR

Forward Primer 5'- CTGGGCAGAAGTTCAACAG -3'
Reverse Primer 5'- AGAAGCGTGTGAGGATAGG -3'

Oligonucleotides for human p87-specific RT-PCR

Forward Primer 5'- TGGTCATTGCCGAACAGAAC -3'
Reverse Primer 5'- TCAGTGGACAGCACAGAAAC -3'

Oligonucleotides for murine p87-specific RT-PCR
Forward Primer 5'- CTGGAGAAGGCAGAAAGC -3'
Reverse Primer 5'- AGGCGACTGTAGAGTAGG -3'

2.1.6 Inhibitors

- Pertussis toxin (ALEXIS, Grünberg)
- LY294002 (Calbiochem, Schwalbach)
- Wortmannin (Sigma Chemical CO., Deisenhofen)
- TGX-221 (Australian Centre for Blood Diseases, Monash University, AMREP, Melbourne, Victoria, Australia)
- AS-252424 (Australian Centre for Blood Diseases, Monash University, AMREP, Melbourne, Victoria, Australia)
- Phenylmethylsulfonylfluoride (PMSF, Sigma Chemical CO., Deisenhofen)
- Protease inhibitor cocktail (PIC) (Roche, Basel, Switzerland)
- Nitro-L-arginine methyl ester (L-NAME), (ALEXIS, Grünberg)

2.1.7 Cell preparation and culture reagents

- Dulbecco's modified Eagle medium (DMEM) (Life Technologies GmbH, Eggenstein)
- DMEM/F-12 (PAA Laboratories GmbH, Pasching, Austria)
- Medium 199 (M199), (Cambrex Bio Science, Verviers SPRL, Belgium)
- Fetal calf serum "heat inactivated" (FCS) (Cambrex Bio Science, Verviers SPRL, Belgium)
- Human serum "heat inactivated" (Cambrex Bio Science, Verviers SPRL, Belgium)
- Endothelial cell growth supplement (ECGS), (Sigma Chemical CO., Deisenhofen)
- Endothelial mitogen (Biomedical Technologies, Stoughton, USA)
- Heparin-sodium (Roche, Basel, Switzerland)
- Heparin (Sigma Chemical CO., Deisenhofen)

- L-Glutamine (ICN Biomedicals, Eschwege)
- Penicillin/Streptomycin (Gibco BRL, Eggenstein)
- Collagenase II (Worthington Biochemical Corporation, Lakewood, USA)
- Trypsin-EDTA (Sigma Chemical CO., Deisenhofen)
- Gelatin (ICN Biomedicals, Eschwege)
- M-450 sheep anti-rat beads (Dynal Biotech, Hamburg)

2.1.8 Other reagents and materials

- Triton X-100 (Carl Roth GmbH & Co KG, Karlsruhe)
- Tween 20 (Serva, Heidelberg)
- Dimethyl sulfoxide (DMSO), (Sigma Chemical CO., Deisenhofen)
- D-Erythro sphingosine 1-phosphate (S1P) (Biomol, Plymouth)
- Bovine serum albumin (BSA, Carl Roth, Karlsruhe)
- Human serum albumin (HSA, Bayer Vital, Leverkusen)
- Prestained protein marker (New England Biolabs, Frankfurt)
- 100bp smart ladder (Eurogentec, Seraing, Belgium)
- ECL^{TM} Western blotting detection reagents (GE Healthcare Lifescience, Buckinghamshire, United Kingdom)
- Hematoxylin (Dako, Hamburg)
- Eukitt mounting medium (Merck, Darmstadt)
- Glutathione-sepharose 4B (Amersham Pharmacia, Uppsala, Sweden)
- Protein A-sepharose (Amersham Pharmacia, Uppsala, Sweden)
- Trizol reagent (Invitrogen GmbH, Karlsruhe)
- Isopropanol (Sigma Chemical CO., Deisenhofen)
- Chloroform (Sigma Chemical CO., Deisenhofen)
- Qiagen one-step RT-PCR kit (Qiagen, Düsseldorf)
- Magnetic particle concentrator (MPC) magnet (Dynal Biotech, Hamburg)
- Mesh (100 µm) (BD Bioscience, Heidelberg)

- Falcon cell culture inserts (0.8 µm)/Companion Plates (BD Bioscience, Heidelberg)
- 0.45 µm Filtropur filtration unit (Sarstedt, Nuembrecht)
- Hoefer electrophoresis chamber (Hoefer Scientific, San Francisco)
- Whatman 3MM paper (Whatman GmbH, Dassel)
- Immobilon polyvinyldifluoride (PVDF) membrane (Millipore Corporation, Bedford, USA)
- BioMax MR-1 film (Eastman Kodak Company New Haven, USA)
- HUVEC nucleofector kit (Amaxa, Koeln)
- SuperFrost slides and coverslips (Menzel, Braunschweig)
- Zeiss inverted microscope (Zeiss, Jena)
- Zeiss laser scanning microscope (Zeiss, Jena)

2.2 Methods

2.2.1 Human umbilical vein endothelial cell (HUVEC) preparation and culture

HUVEC were isolated using 0.1 % collagenase and cultured on 0.2 % gelatin-coated 75-cm^2 flasks in M199 supplemented with 20 % heat-inactivated serum (15 % FCS, 5 % human serum), 7.5 µg/ml endothelial cell growth supplement (ECGS), 7.5 U/ml heparin-Na, 100 U/ml penicillin-streptomycin. Cells were maintained at 37°C in 5 % CO_2. Confluent cultures were detached by brief treatment with trypsin-EDTA (0.05 %/0.02 %) and plated onto 60-mm, 90-mm dishes, or tissue culture inserts for stimulation and migration experiments. All experiments were performed with HUVEC monolayers of the second passage.

2.2.2 Mouse lung endothelial cell (MLEC) preparation and culture

Isolation of cells from murine lung

MLEC from wild type and PI3Kγ knockout mice were prepared using a modified method from Kuhlencordt et al. (2004). Briefly, animals were sacrificed by cervical dislocation and soaked in 70 % ethanol. Their lungs were collected in 20 ml of ice-cold DMEM. After removing the hilus region from each lobe, the lungs were minced into small pieces under the laminar flow cabinet and digested with 0.1 % collagenase A for 1 h at 37°C. The digest was triturated through a blunt 14-gauge needle and passed through a 100-µm plastic mesh. Cells were pelleted at 300 x *g* for 10 min and resuspended in 15 ml of culture medium, called “plus medium” (preparation see below). The cell suspension was plated in a T75 flask precoated with 0.1 % gelatin. The next day, cells were washed twice with 5 ml phosphate buffered saline (PBS) containing 2 % FCS and cultured for 4 - 5 days. 15 ml of fresh “plus medium” was replaced every other day.

Selection of endothelial cells from the cell culture

Endothelial cells were selected by sheep anti-rat antibody pre-coated magnetic beads, which were additionally incubated with an endothelial-specific antibody (Fig. 5). For this purpose, $2x10^6$ magnetic beads per flask were washed three times for 2 min with 1 ml PBS/2 % FCS and were subsequently incubated with 7.5 µg of purified rat anti-mouse CD102 antibody in 500 µl PBS/2 % FCS on a rotator for 2 h at 4°C. Using a neodymium-iron-boron permanent magnet, beads were harvested and rinsed with 1 ml PBS/2 % FCS three times, 2 min each.

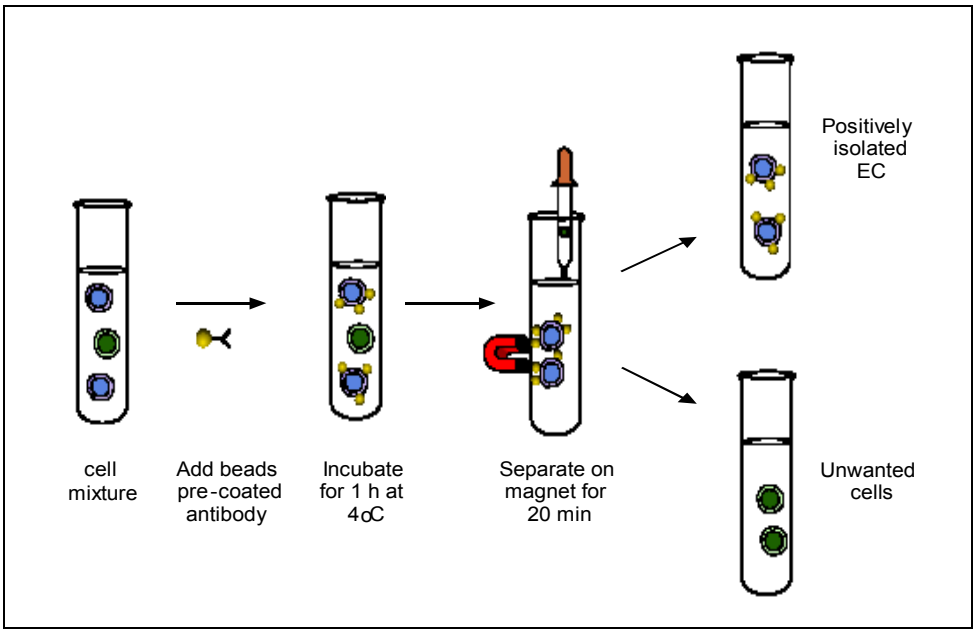

Figure 5. Magnetic selection
(Adapted from Dynal product brochure)

Antibody-coated beads were resuspended with fresh PBS/2 % FCS buffer. $2x10^6$ magnetic beads were added to each cell culture flask and incubated for 1 h at 4°C, thus allowing binding of antibody-coated beads to endothelial cells. Incubation medium was then removed by aspiration and the flask was rinsed with 2 ml trypsin-EDTA. Cells were detached by trypsinisation with 2 ml of fresh solution.

Reaction was stopped by addition of 2 ml of “minus medium” (preparation see below). Cells suspension in a total volume of 15 ml of “minus medium” were transferred to a 15-ml Falcon tube and endothelial cells were selected in a magnetic field for 10 min. To avoid detachment of beads-bound cells that stuck on the wall of the tube, the Falcon tube was kept on the magnet and the medium containing unwanted cells was gently poured out. The selected endothelial cells were then resuspended in fresh “plus medium” and were cultivated in the gelatin-precoated T75 flask containing 15 ml of “plus medium” at 37^{o}C, 5 % CO_2. When cells were confluent, selection was repeated once before plating cells for experiments. By following this procedure, cells used in the experiments were, on average, 2 weeks in culture.

- Minus medium:

35 %	DMEM
35 %	F12
20 %	FCS
2 mM	L-glutamine
100 U/100 µg/ml	penicillin-streptomycin

- Plus medium:

40 %	DMEM
40 %	F12
20 %	FCS
2 mM	L-glutamine
50 µg/ml	endothelial mitogen
25 µg/ml	heparin
100 U/100 µg/ml	penicillin-streptomycin

- PBS:

13.6 mM	NaCl
2.7 mM	KCl
8.1 mM	Na_2HPO_4
1.8 mM	KH_2PO_4
pH	7.5

2.2.3 Immunohistochemistry

HUVEC and MLEC were stained by using the “ABC staining” system. Briefly, cells were cultured on glass coverslips precoated with 0.2 % gelatin in a 24-well plate till they were confluent. On the day of experiment, cells were washed twice with Hepes-Ca buffer and fixed with 4 % paraformaldehyde (PFA)/PBS for 10 min. The coverslip was rinsed twice with PBS buffer, 5 min each, and incubated for 1 h with 50 µl of anti-mouse or anti-human CD31 antibody (1:50 diluted in PBN buffer). The primary antibody was removed by three washes with PBS and the coverslip was incubated for 30 min with 50 µl of biotin-conjugated secondary antibody (1: 50 diluted in PBN buffer). Afterwards, cells were rinsed three times with PBS, incubated with 50 µl of streptavidin-conjugated horseradish-peroxidase solution for 30 min and washed additionally three times for 2 min with PBS. 50 µl of peroxidase substrate solution supplied with 2 µl of 3,3’ diaminobenzidine (DAB) chromogen was given to the coverslip and incubated for 10 min. The coverslip was then rinsed with tap water, counterstained with hematoxylin for 30 sec and cleared with tap water three times. After immediately adding 2 - 3 drops of mounting medium to the coverslip, cells were observed by light microscopy and photographed. All procedures were performed at room temperature and coverslips were kept wet during the experiment.

- Hepes-Ca buffer:

10 mM	Hepes-Na
145 mM	NaCl
5 mM	KCl
1 mM	$MgSO_4$
10 mM	Glucose
1.5 mM	$CaCl_2$
pH 7.4	

- PBN buffer:

1x	PBS
0.1 %	BSA
0.1 %	Na_3N_3

2.2.4 Endothelial cell transfection

Nucleofection

Endothelial cells were transfected using the AMAXA Biosystems nucleofection procedure and according to the manufacturer's instructions (www.amaxa.com). Briefly, cells were washed twice with Hepes buffer and detached using warm trypsin-EDTA solution. Trypsinisation was terminated with M199/10 % FCS. Cells were pelleted by centrifugation (500 x g, 6 min), resuspended in Hepes-Na buffer and counted in a Neubauer hemacytometer. For each transfection, 1 x 10^6 cells were used. The required number of cells was pelleted by centrifugation (500 x g, 6 min) and resuspended in HUVEC nucleofector solution (100 µl per transfection). The cell suspension was mixed with plasmid DNA (2 µg/100 µl) and transferred to an electroporation cuvette. Electroporation was carried out using the nucleofection program U-01. Immediately after electroporation, cells were covered with 400 µl warm growth medium and plated onto 60-mm dishes containing 3 ml of growth medium. After six hours, growth medium was replaced. Cells were analysed for protein expression or harvested for cellular assays 24 h post-transfection.

- Hepes-buffer:

10 mM	Hepes-Na
145 mM	NaCl
5 mM	KCl
1 mM	$MgSO_4$
10 mM	Glucose
pH 7.4	

2.2.5 Cell stimulation and cell lysis

HUVEC were seeded onto 60-mm or 90-mm dishes and were washed once and serum-starved with M199/ HSA (fatty acid-free) for 5 h. Stimulation was then

performed in Hepes-Ca buffer containing 0.25% HSA. Thereafter, cells were washed with cold wash buffer and immediately placed on ice. Cells were kept in cold lysis buffer (100 µl/60-mm dish) on ice for 15 min and then scraped from the dish using a cell scraper. Lysates were collected in eppendorf tubes, sonicated and centrifuged (3,000 x g, 6 min, 4°C) to remove the Triton-insoluble material. The cleared lysates were transferred to new tubes and 10 µl was set aside for determination of protein concentration. Lysates were diluted with 3-fold concentrated Laemmli buffer, heated at 95°C for 5 min, and stored at -20°C until use. Protein concentration was determined using the Lowry method (Biorad DC Assay) and bovine serum albumin as standard.

- Wash buffer:

50 mM	Tris, pH 7.4
2 mM	EDTA
1 mM	EGTA
50 mM	NaF
10 mM	$Na_4P_2O_7$*
1 mM	DTT*

(* freshly added)

- Lysis buffer:

1x	washing buffer
0.1 %	SDS*
1 %	Triton X-100*
10 µl/ml	PIC*
1mM	PMSF*

(* freshly added)

- Laemmli buffer (3X):

186 mM	Tris, pH 6.8
10 mM	EDTA
9 %	SDS
15 %	Glycerol
6 %	β-Mercaptoethanol
0.03 %	Bromophenol Blue

2.2.6 Immunoprecipitation

Protein A-sepharose slurry was washed three times with 500 µl cold lysis buffer. To decrease unspecific binding to the beads during incubation with specific antibodies,

lysate protein was precleared by adding protein A-sepharose beads. After incubation on a rotator at 4°C for 30 minutes, beads were removed by centrifugation (20 000 x g, 4°C, 5 min). 4 - 12 µg of PI3Kβ-specific antibody was added to each tube containing 250 - 750 µg of precleared lysate and the mixture was gently rotated overnight at 4°C. Immunocomplexes were precipitated by adding 2.5 - 7.5 mg of protein A-sepharose slurry. After 1 h incubation on the rotator at 4°C, beads were collected by centrifugation at 4°C, 20 000 x g, 2 min, and washed twice with 500 µl of cold lysis buffer. To dissociate the immunocomplex from the beads, the supernatant was carefully aspirated and the pellet was boiled for 5 min with 60 µl of 3-fold Laemmli sample buffer at 95°C. After cold centrifugation the supernatant was transferred in a new Eppendorf tube and frozen at -20°C. Using SDS-PAGE, proteins were separated and detected by Western blotting.

2.2.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The prepared samples were loaded onto an SDS-PAGE gel and an electrical field was applied. Thereafter, the proteins were transferred to PVDF membranes using a semi-dry transfer apparatus. Briefly, the sandwich was prepared and the blotting was performed at a set current of 1.5mA/cm^2 for 2.5 h. Afterwards, the PVDF membrane was washed briefly in TBS/Tween-20 solution (TBS-T) and blocked with 5 % nonfat dry milk in TBS-T for 1 h. Blots were probed with the appropriate primary and secondary antibodies in 1 % bovine serum albumin/TBS-T solution overnight or for 1 h, respectively, and detection was carried out using ECL chemiluminescence reagent (Amersham) and exposure onto BioMax film (Kodak). For counterstaining, immunoblots were stripped by incubating blots in stripping buffer at 56 °C for 30 min, with agitation and reprobed with appropriate antibodies.

• TBS (10X):	137 mM	NaCl
	2.68 mM	KCl
	25 mM	Tris base
	pH 7.4	
• TBS-Tween (TBS-T):	1X	TBS
	0.1 %	Tween-20
• Electrophoresis buffer:	25 mM	Tris
	250 mM	Glycine (pH 8.3)
	0.1 %	SDS
• Transfer buffer:	48 mM	Tris base
	39 mM	Glycine
	0.037 %	SDS
	20 %	Methanol
• Stripping Buffer:	62.5 mM	Tris-HCl, pH 6.7
	2 %	SDS
	0.7 %	β-mercaptoethanol

2.2.8 Rac1 GTPase activation assay

The activation of Rac was measured in a pull-down assay, in which the CRIB (Cdc42- and Rac-interacting binding) domain of p21-activated kinase (PAK) was used to isolate the active GTP-bound form of Rac (Bagrodia et al., 1995). A glutathione S-transferase (GST)-tagged PAK CRIB domain was employed in pull-down assays. Briefly, cells were seeded on 0.2 % gelatin-coated 90-mm dishes and grown until 90 - 95 % confluence. On the day of experiment, cells were serum-starved for 5 h and pretreated with or without inhibitors during the last 30 min and stimulated with S1P (1 µM, 1 min). Cells were placed on ice immediately and lysed in cold lysis buffer containing GST-PAK (20 µg/ml), protease inhibitors (10 µl PIC/ml) and GDP (100 µM). Lysates were collected, vortexed for 10 sec and cleared by centrifugation at 4°C, 20.000 x g, 15 min. Protein concentration determination was performed and 200 µg of

proteins were transferred to a new tube. GSH-sepharose beads were washed three times in 500 µl lysis buffer and 1:1 suspended with cold lysis buffer. 40 µl of this suspension was given to each protein sample and incubation was performed on a rotator for 30 min, 4°C. Afterwards, the beads were centrifuged, washed three times with cold lysis buffer, resuspended with 3-fold concentrated Laemmli buffer and heated at 95°C for 5 min. Beads were removed by brief centrifugation at 4°C, 20.000 x g. Supernatants were transferred to new tubes and cleared by centrifugation once more. Samples were separated by 15 % SDS-PAGE, transferred to PVDF membranes and immunoblotted using anti-Rac1 monoclonal antibody (1:1000 diluted in 5 % BSA/TBS-T). To avoid transfer of the GST-PAK protein (approx. 30 kDa), gels were cut slightly below the 30 kDa band of marker and only the lower part was transferred and immunolblotted against Rac1 (21 kDa).

- Lysis Buffer:

50 mM	Tris, pH 7.4
50 mM	NaCl
5 mM	$MgCl_2$
1 mM	EGTA
1 %	NP-40
10 %	Glycerol
10 µl/ml	PIC *
100 µM	GDP *
20 µg/ml	GST-PAK *

* (freshly added)

2.2.8 Migration assay

Cell migration assays were performed using tissue culture inserts (polyethylene terephthalate (PET) membrane, 8 µm pores; BD Falcon Inserts) situated in 12-well plates. Inserts were coated with 0.2 % gelatin solution overnight at 4 °C. On the day of experiment, the gelatin solution was removed by aspiration and inserts were allowed to dry. HUVEC grown to sub-confluency.were washed twice with pre-warmed Hepes-Ca buffer and detached using trypsin-EDTA solution. Trypsinisation was stopped by M199/10 % FCS. Cells were pelleted by centrifugation (500 x g, 6 min)

and resuspended in M199/0.25 % HSA. The suspension was diluted to 5 x 10^5 cells/ml and 0.5 ml (2.5 x 10^5 cells) was applied to the upper chamber of the insert. To the lower chamber, 1 ml of M199/0.25 % HSA was added. Inhibitors were applied to both chambers and incubated for 30 min. Finally, 1 µM S1P was added to the lower chamber and the migration apparatus was gently agitated to ensure even distribution of the chemoattractant. Migration was allowed to proceed for 4 h at 37°C and 5 % CO_2.

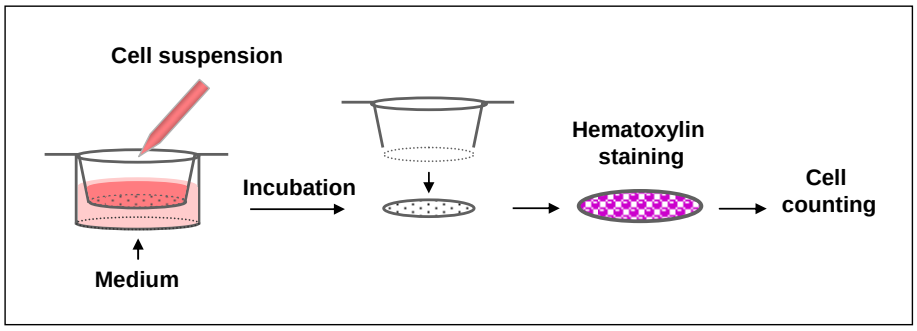

Figure 6. Transwell plate Migration assay

Hematoxylin Staining

Cells were washed twice using ice-cold Hepes-Ca buffer and fixed with 4 % paraformaldehyde in PBS for 10 min. The fixing solution was removed by aspiration and inserts were washed twice with double distilled H_2O. Inserts were stained with hematoxylin for 5 min, washed twice with tap water and incubated for 10 min to allow color development. Afterwards, the tap water was removed by aspiration and the non-migrated cells were removed by wiping the upper face of the insert with a cotton-tip. Inserts were allowed to air-dry overnight in the dark. Using a clean scalpel, inserts were carefully excised from their support and embedded onto tissue culture slides using Eukitt mounting medium. The number of migrated cells was counted using an inverted microscope (Zeiss) at 25-fold magnification and determined as the mean of 10 randomly selected fields.

2.2.9 Wound Healing Assay

Endothelial cells were seeded on 6-well plates coated with 0.2 % gelatin and allowed to achieve confluence. Subsequently, one 1 - 2 mm wide wound area was scratched into the monolayer using a 1-ml pipette tip and the position of the denuded area was indicated with the help of an ocular grid. Medium was aspirated and dishes were rinsed once with PBS. Cells were then incubated with inhibitors for 30 min in 2 ml of fresh M199/5 % FCS and subsequently stimulated with 1 µM S1P for 20 h. To quantitate the level of cell migration, cells which migrated into the marked areas were counted (10 fields per well) using a light microscope.

2.2.10 Total RNA isolation

Total RNA was isolated from HUVEC and MLEC using Trizol reagent. Cells were lysed directly in a 90-mm culture dish by adding 1.6 ml of Trizol reagent, distributing the reagent evenly and passing the cell lysate several times through a pipette. The homogenised samples were collected in a 2-ml tube and incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml/ml of chloroform was applied to the samples and mixed for 15 sec. After 3 min incubation, samples were centrifuged at 12.000 x g, 4°C for 15 min. The colorless upper aqueous phase which contains RNA was carefully transferred to a new tube. RNA was precipitated by adding 0.5 ml/ml of isopropanol and collected by centrifugation (12.000 x g, 10 min 4°C). The pellet was washed with 1.5 ml of cold 75% ethanol. After centrifugation (7500 x g, 5 min, 4°C) the pellet was allowed to dry at room temperature. Finally, the RNA sample was dissolved in 50 µl diethyl pyrocarbonate (DEPC) water and stored at -20°C until use. The concentration of RNA was determined as the absorbance at 260 nm in spectrophotometer, where 1 unit at 260 nm corresponds to 40 µg of RNA per ml.

2.2.11 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the Qiagen OneStep RT-PCR Kit (QIAGEN) to detect mRNA expression of p101 and p87 in HUVEC and MLEC. With the kit, reverse transcription and polymerase chain reaction was allowed to take place sequentially in the same tube. For each reaction, 1.0 μg RNA and 500 nM forword/reverse primers were used. The conditions of reaction are shown as below:

Human p101- or p87-specific RT-PCR

•	Reverse transcription:	30 min	50°C
•	Initial PCR acivation:	15 min	95°C
•	3-step cycling:		
	- Denaturation:	1 min	94°C
	- Annealing:	1 min	55°C
	- Extension:	1 min	72°C
	- Number of cycles:	30 cycles	
•	Final extension:	10 min	72°C

Mouse p101- or p87-specific RT-PCR

•	Reverse transcription:	30 min	50°C
•	Initial PCR acivation:	15 min	95°C
•	3-step cycling:		
	- Denaturation:	1 min	94°C
	- Annealing:	1 min	55°C
	- Extension:	1 min	72°C
	- Number of cycles:	35 cycle	
•	Final extension:	10 min	72°C

PCR reaction products were mixed with 5-fold concentrated DNA loading buffer and separated by electrophoresis on 1.0 % agarose gel in Tris-acetate buffer (TAE buffer) containing 0.5 μg/ml ethidium bromide and visualised using a UV illuminator.

- 5× DNA sample buffer: 0.25% Bromphenol blue
  0.25% Xylencyanol
  30% Glycerol
- 1x TAE buffer: 40 mM Tris-acetic acid
  1 mM EDTA
  pH 8.3

2.2.12 Statistical analysis

All data are given as means ± SEM of three to five independent experiments. To determine the statistical significance of the described results, analysis of variance with Bonferroni's correction for multiple comparisons, or t-tests were performed. A p value of < 0.05 was accepted as statistically significant.

3. Results

3.1 Characterisation of endothelial cells

Our studies were performed in HUVEC and MLEC from wild type and PI3Kγ knockout mice which lack the catalytic subunit p110γ. $p110\gamma^{-/-}$ mice were generated a few years ago (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000), while the p110β knockout mouse is not available. To characterise human and particularly murine endothelial cells, they were specifically labeled with anti-CD31 human- or mouse-specific antibodies using the "ABC staining" system. As shown in Fig. 7, most cells showed positive staining compared to control cells which were not incubated with the anti-CD31 antibody.

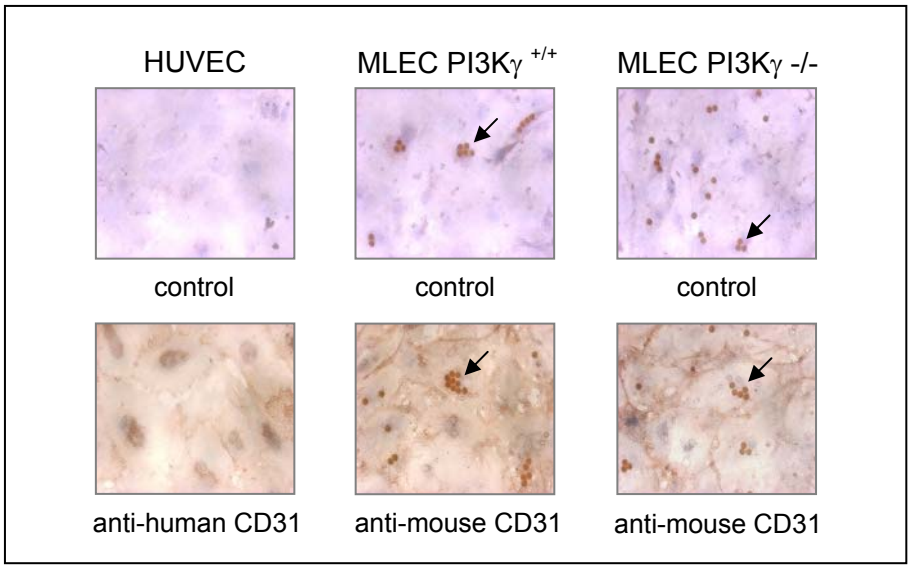

Figure 7. Photomicrographs of human or mouse-specific CD31 immuno-staining. HUVEC and MLEC were seeded on gelatine-coated coverslips situated in 24-well plates. Cell monolayer was stained without (upper lane) or with (lower lane) anti-CD31 antibody. Nuclei were stained with Hematoxylin. Arrows point to the magnet beads from mouse lung endothelial cell preparation.

3.2 PI3K expression in endothelial cells

3.2.1 Expression of catalytic subunit of endogenous PI3K isoforms in HUVEC and MLEC

PI3K class I is subgrouped into class IA and class IB. Class IA contains three isoforms p110α, β, and δ, whereas p110γ has been characterised as the unique isoform of PI3K class IB that is activated by GPCR. Its expression has been initially believed to be restricted to cells of the haematopoietic lineage. Recent findings indicated the existence of PI3Kγ in smooth muscle cells (Bacqueville et al., 2001) and endothelial cells (Puri et al., 2005) suggesting that PI3Kγ possesses a broader distribution pattern than previously thought.

In the present study, we investigated the presence of the PI3Kγ catalytic subunit, p110γ, in HUVEC and MLEC using Western blot analysis. Fig. 8 confirms the existence of p110γ in human and wild type murine endothelial cells at the protein level using a specific antibody against the N-terminus of p110γ. On the other hand, as expected, the protein expression of p110γ was not detectable in cells that were isolated from p110γ-null mice (Fig. 8).

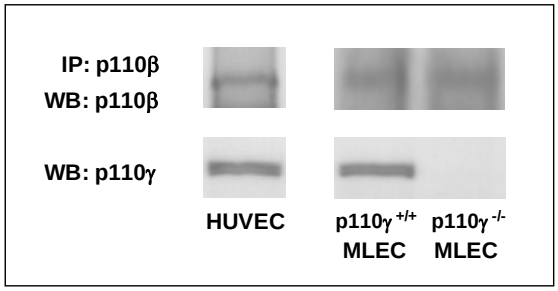

Figure 8. Expression of p110γ and p110β in endothelial cells. HUVEC or MLEC were cultured on 60 mm-dishes and lysed with lysis buffer. PI3Kβ-immunocomplexes or protein lysates (PI3Kγ) were separated by SDS-PAGE on 10 % gels. PI3K isoforms were detected by immunoblotting using specific antibodies against p110β or p110γ. Protein expression in HUVEC, wild type and p110γ knockout MLEC is shown.

PI3Kβ is another member of class I PI3Ks which is partially activated by GPCR. We also investigated its presence in human and mouse endothelial cells by employing an antibody specifically directed to p110β to immunoprecipitate the protein and to identify its expression in Western blots. Fig. 8 shows the expression of PI3Kβ in HUVEC and MLEC. Interestingly, the absence of PI3Kγ protein in knockout MLEC had no effect on the protein level of PI3Kβ.

3.2.2 Expression of regulatory subunits of PI3Kγ in HUVEC and MLEC

Several years ago, a protein, termed p101, has been investigated and described as an essential adaptor protein of PI3Kγ (Stephens et al., 1997). More recent studies depicted the presence of another regulatory subunit of PI3Kγ, p87, in human embryonic kidney epithelial cell line, HEK293 (Voigt et al., 2006). We also investigated whether these adaptors are expressed in human and murine endothelial cells. Since specific antibodies are not available to detect protein expression, we examined the expression of p101 and p87 at the mRNA level. According to human or murine sequences of p101 and p87, oligonucleotide primers were designed and employed in RT-PCR as described. The expected products corresponding to different size (human p101: 481 bp, human p87: 607 bp; mouse p101: 405bp, mouse p87: 169 bp) were separated and detected by 1% agarose gel electrophoresis. A human T cell line, Jurkat, and a murine myeloid cell line, CD32, both expressing p101 and p87, were employed as positive controls for human and mouse samples, respectively.

Neither p101 nor p87 mRNA was detected in HUVEC, although the expression of both adaptors was clearly seen in control cells (Fig. 9). In contrast, it is interesting to note that MLEC showed the presence of both p101 and p87, suggesting that the expression of both regulatory subunits may depend on the cell type and/or species.

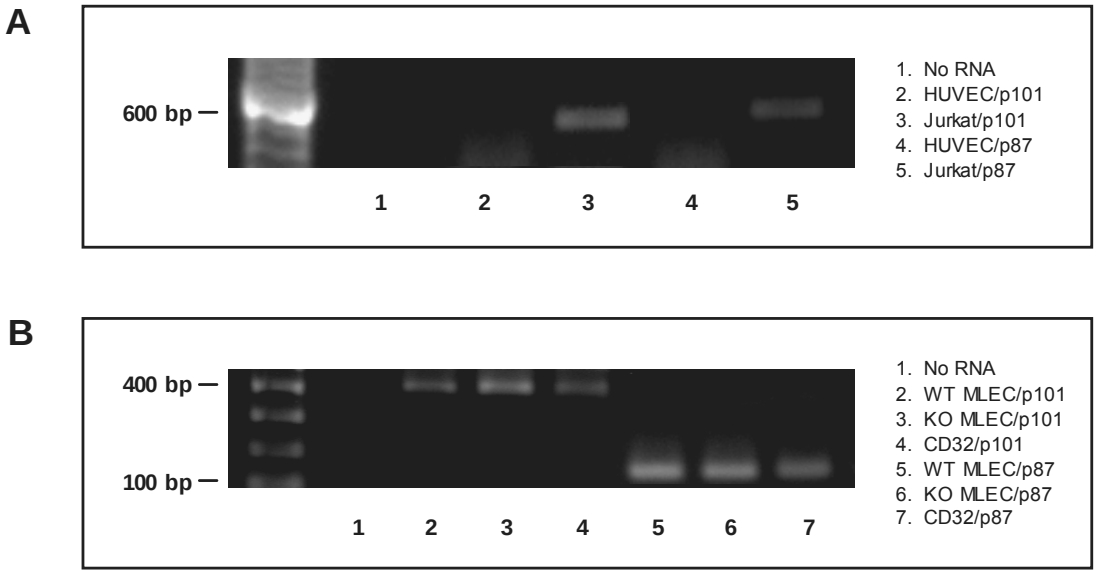

Figure 9. mRNA expression of PI3Kγ regulatory subunits p87 and p101 in human (A) and murine (B) endothelial cells. Total RNA was isolated from HUVEC or normal and p110γ knockout MLEC. RT-PCR was performed using the Qiagen OneStep RT-PCR Kit (0.5 µg RNA and 50 pmol forward/reverse primers). RT-PCR products were separated on 1% agarose gels containing 0.5 µg/ml ethidium bromide and visualised using UV light. Samples prepared from Jurkat and CD32 cell lines were used as positive control for human and mouse cells, respectively.

3.3 S1P-induced protein phosphorylation in endothelial cells

3.3.1 Effect of PI3K inhibitors on S1P-induced phosphorylation of Akt in HUVEC

Akt is an essential signalling protein involved in the regulation of cellular responses such as cell survival and migration. Many agonists are able to influence Akt activation by regulating its phosphorylation state. In the present study, we show that S1P triggers a rapid, transient and reversible Akt phosphorylation in HUVEC. Using a specific antibody against the phosphorylated serine 473 residue of Akt, a time-dependent Akt phosphorylation in response to stimulation with 1 µM S1P was detected from 15 sec to 10 min with the peak observed at 2 min (Fig.10).

Previous reports have demonstrated that S1P is able to stimulate Akt activation via a

G_i/PI3K signalling pathway in many cell types. In this study, HUVEC were pretreated with the $G_{i/o}$ protein inhibitor PTX and the PI3K inhibitor wortmannin which restrains all members of class I PI3Ks. As presented in Fig. 11, the level of phosphorylated Akt following S1P stimulation (1 µM, 2 min) was markedly reduced by PTX (100 ng/ml, 3 h) or wortmannin (100 nM, 30 min) treatment, confirming earlier findings that both G_i protein and PI3K are required for S1P-mediated Akt activation.

Since we were able to demonstrate that both PI3Kβ and PI3Kγ are expressed in HUVEC and since both isoforms have been described to be targets of Gi protein βγ subunits, we investigated whether one or both of these PI3K isoforms mediate S1P-induced Akt phosphorylation.

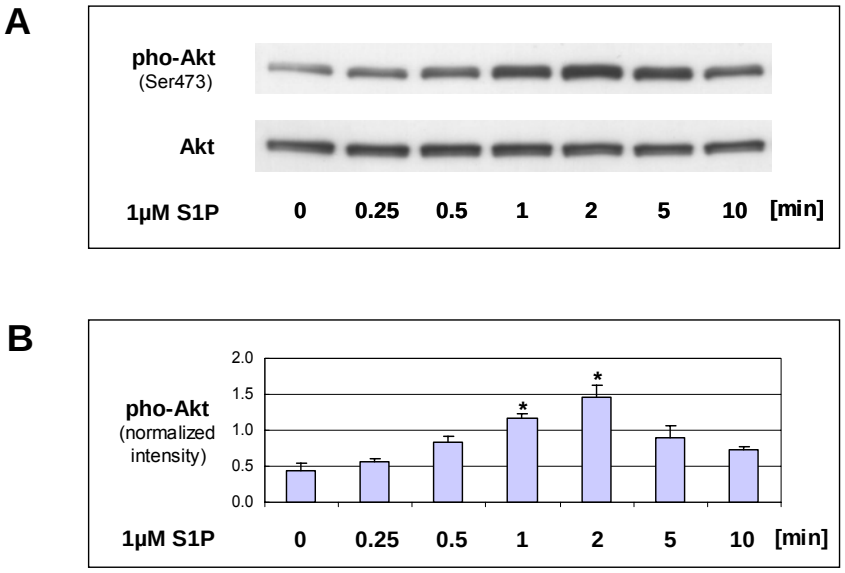

Figure 10*. Time-dependency of S1P-induced Akt phosphorylation in HUVEC. HUVEC were serum starved with M199/0.25 % HSA for 5 h in 60-mm dishes and subsequently stimulated with 1µM S1P for different time points (0, 0.25, 0.5, 1, 2, 5, 10 min). Lysis buffer was directly applied into dishes and the lysates were collected by centrifugation. **(A)**. Lysates were analysed by immunoblotting using a specific antibody against phosphorylated Akt (serine 473) or, after stripping, total Akt. One of three similar blots is shown. **(B)**. Densitometry analysis of pooled data showing the ratio between phosphorylated Akt and total Akt after S1P stimulation. The data represent mean values ± SEM derived from three independent experiments.*p < 0.05 versus untreated control.
(* Experiments were performed by Gunter Ehrlich)

Therefore, HUVEC were pretreated with distinct inhibitors TGX-221 and AS-252424, which specifically inhibit the activation of PI3Kβ and PI3Kγ, respectively. In Fig. 11, we present the interesting result that treatment of cells with the PI3Kβ-specific inhibitor TGX-221 (100 nM, 30 min) completely abrogated S1P (1 µM, 2 min)-mediated Akt phosphorylation which was comparable to the effects of PTX and wortmannin. Surprisingly, in the same studies, no alteration of S1P-induced Akt activation was observed when cells were preincubated with the PI3Kγ-specific inhibitor AS-252424 (1 µM, 30 min). These results indicated the requirement of PI3Kβ, but not PI3Kγ, for S1P-mediated activation of Akt in human endothelial cells.

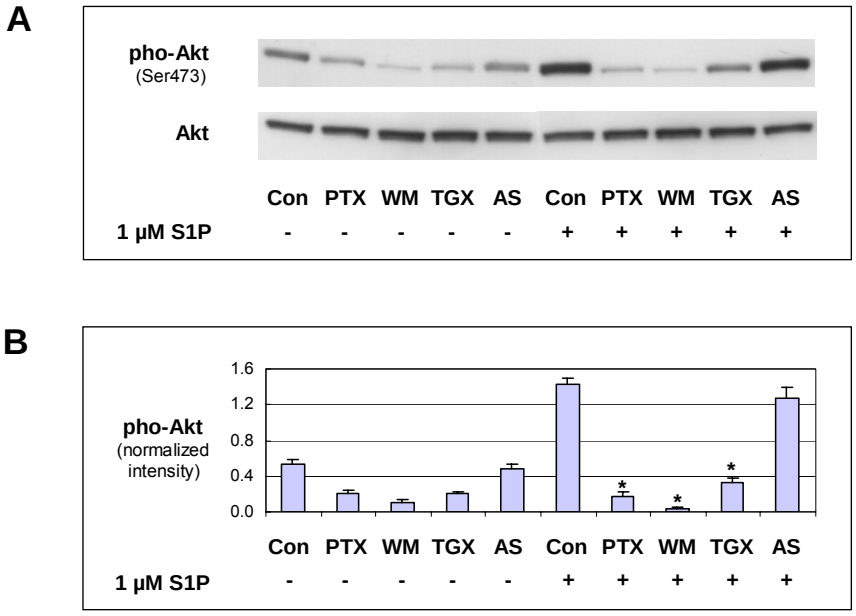

Figure 11*. Effect of G_i protein and PI3K inhibitors on S1P-stimulated Akt phosphorylation in HUVEC. HUVEC were serum starved with M199/0.25 % HSA for 5 h in 60-mm dishes and pretreated with different inhibitors for 30 min (100 nM wortmannin (WM), 100 nM TGX-221 (TGX), 1 µM AS-252424 (AS)) or 3 h (100 ng/ml pertussis toxin (PTX)). Subsequently, stimulation with 1µM S1P for 2 min was performed. **(A)**. Cells were lysed in lysis buffer and the lysates were analysed by immunoblotting using a specific antibody against phosphorylated Akt (serine 473) or, after stripping, total Akt. One of four similar blots is shown. **(B)**. Densitometry analysis of pooled data showing the ratio between phosphorylated Akt and total Akt after S1P stimulation. The data represent mean values ± SEM derived from four independent experiments. *p < 0.05 versus S1P-stimulated control. Con, control.
(* Experiments were performed by Gunter Ehrlich.)

3.3.2 S1P-induced phosphorylation of Akt in MLEC

PI3Kγ knockout mice have been proved to be an excellent tool to study the function of PI3Kγ in biological responses. With the respect to its role in Akt activation, some groups have shown that Akt phosphorylation induced by GPCR-coupled agonists,

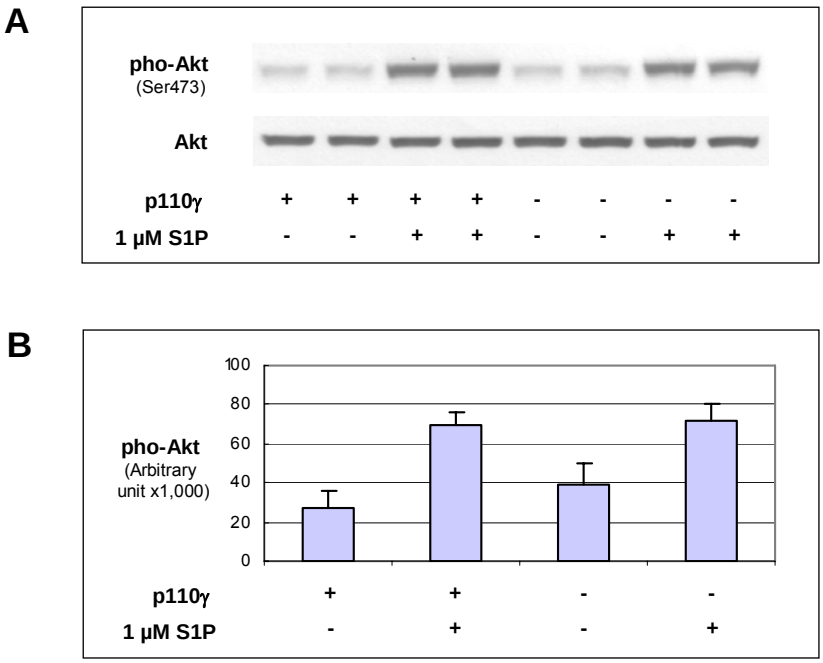

Figure 12. S1P-induced Akt phosphorylation in wild type and p110γ knockout MLEC. Wild type and knockout MLEC were serum starved for 5 h and stimulated with 1µM S1P for 2 min. **(A)**. Cell lysates were analysed by immunoblotting using a specific antibody against phosphorylated Akt (serine 473) or, after stripping, total Akt. One of four similar blots is shown. **(B)**. Densitometry analysis of the blots of phosphorylated Akt and total Akt under basal conditions or after S1P stimulation shown in Fig. A. The data represent mean values ± SEM derived from four independent experiments.

such as fMLP, C5a and IL-8, was decreased in p110$\gamma^{-/-}$ neutrophils (Hirsch et al., 2000; Sasaki et al., 2000). In the present study, we addressed whether the inhibition of S1P-mediated Akt phosphorylation can also be observed in p110$\gamma^{-/-}$ endothelial cells. Studies were performed with MLEC isolated from normal and p110γ-deficient mice.

Fig. 12 shows that the absence of PI3Kγ protein in p110γ knockout cells was not able to alter the S1P (1 µM, 2 min)-elicited level of phosphorylated Akt. This finding further confirms our results obtained in inhibitor studies in HUVEC and indicates that PI3Kγ was not required for S1P-induced Akt activation in endothelial cells.

3.3.3 Effect of PI3K inhibitors on S1P-induced phosphorylation of eNOS in HUVEC

eNOS is a well-known downstream effector of Akt in response to many agonists. Rikitake et al. have reported that S1P is also able to phosphorylate eNOS via a G_i protein/PI3K/Akt pathway, thus leading to increased NO production in endothelial cells (Rikitake et al., 2003). However, the PI3K isoform involved was not specified, although Igarashi et al. suggested that PI3Kβ might be activated by S1P (Igarashi et al. 2001). Therefore, we evaluated the effect of distinct PI3K inhibitors on S1P-induced eNOS phosphorylation in HUVEC.

In agreement with previous reports, we show that 1 µM S1P significantly stimulated eNOS phosphorylation at the serine 1177 residue by about 4-fold after 2 min, which was completely inhibited by 100 ng/ml PTX. However, pretreatment of cells with 100 nM wortmannin for 30 min, only partially reduced S1P-induced eNOS phosphorylation (Fig 13), although Akt was completely inhibited under these conditions. Furthermore, we determined TGX-221-induced effects on eNOS phosphorylation. Interestingly, preincubation of cells with 100 nM of the p110β-specific inhibitor TGX-221 for 30 min exhibited only approximately 35 % reduction of eNOS phosphorylation in response to S1P (1 µM, 2 min), while Akt activation was fully abolished in the parallel experiments. Moreover, no alteration of eNOS phosphorylation was detected upon AS-252424 pretreatment (1 µM, 30 min). These results show that PI3Kβ partially mediates eNOS phosphorylation whereas PI3Kγ is not involved. Apart from the PI3K/Akt pathway, however, other protein kinases seem to contribute to S1P-induced eNOS phosphorylation as well.

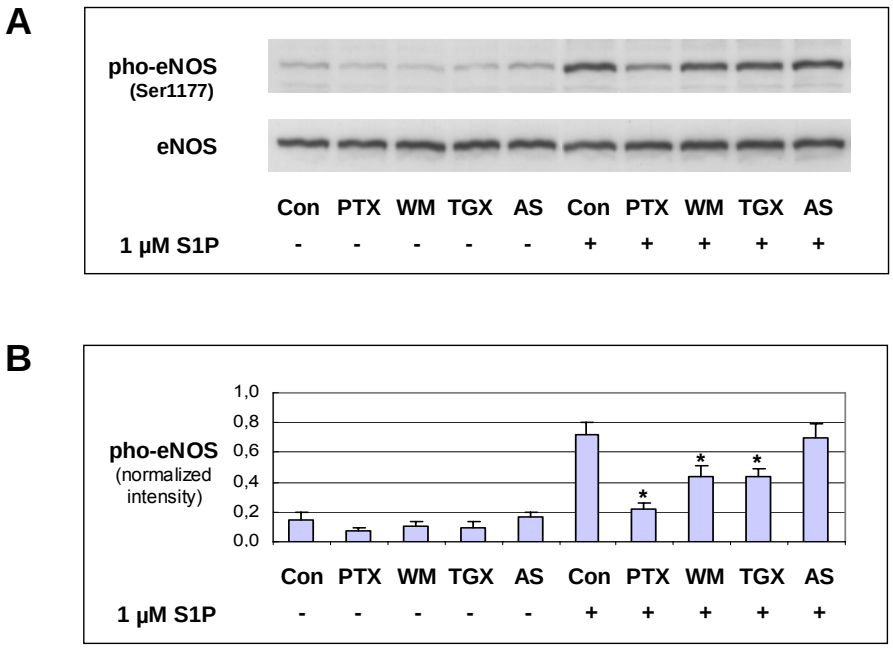

Figure 13*. Effect of G_i protein and PI3K inhibitors on S1P-stimulated eNOS phosphorylation in HUVEC. HUVEC were serum starved with M199/0.25 % HSA for 6 h in 60-mm dishes and pretreated with different inhibitors for 30 min (100 nM wortmannin (WM), 100 nM TGX-221 (TGX), 1 µM AS-252424 (AS)) or 3 h (100 ng/ml pertussis toxin (PTX)). Subsequent stimulation of 1µM S1P was performed for 2 min. **(A)**. Cells were lysed as described and the lysates were analysed with immunoblotting using a specific antibody against phosphorylated eNOS or, after stripping, total eNOS. One of four similar blots is shown. **(B)**. Densitometry analysis of pooled date showing the ratio between phosphorylated Akt and total Akt after S1P stimulation. The data represent mean values ± SEM derived from four independent experiments. *p < 0.05 versus S1P-stimulated control. Con, control.
(* Experiments were performed by Gunter Ehrlich.)

3.3.4 S1P-induced phosphorylation of eNOS in MLEC

S1P-induced eNOS phosphorylation was also analysed in MLEC isolated from p110γ-negative murine cells (Fig. 14). 1 µM S1P (2 min) obviously increased eNOS phosphorylation in knockout MLEC. However, p110γ-negative cells did not show a significant alteration on the level of eNOS phosphorylation compared to that observed in wild type cells (Fig. 14).

These results confirm that S1P stimulates phosphorylation of eNOS partially via a G_i protein/PI3Kβ/Akt signalling pathway, whereas PI3Kγ is not involved.

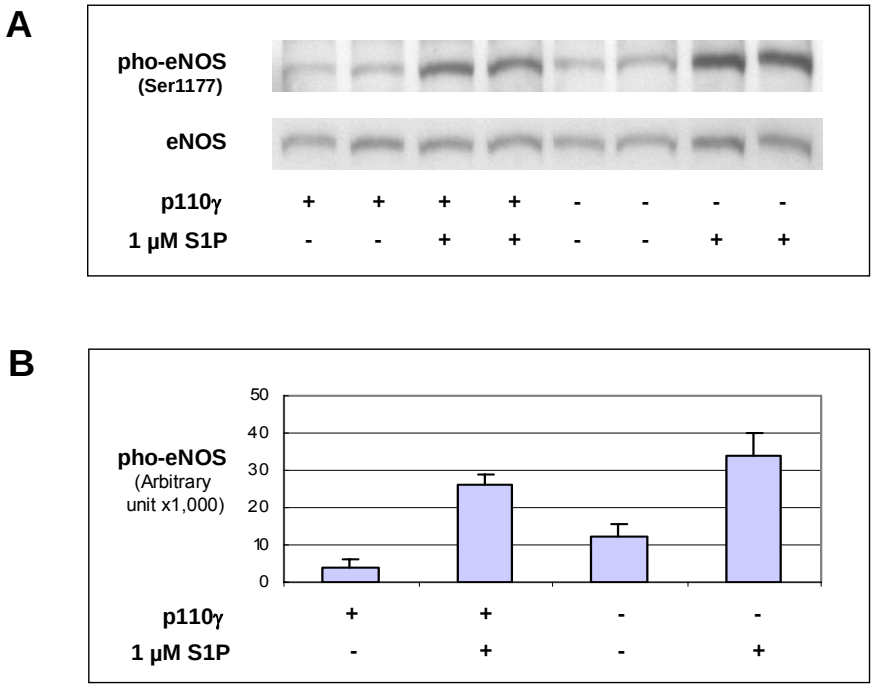

Figure 14. S1P-induced eNOS phosphorylation in wild type and p110γ knockout MLEC. Wild type and p110γ knockout MLEC were serum starved for 5 h and subsequently stimulated with 1µM S1P for 2 min. **(A)**. Cell lysates were analysed by immunoblotting using a specific antibody against phosphorylated eNOS (serine 1177) or, after stripping, total eNOS. One of four similar blots is shown. **(B)**. Densitometry analysis of pooled data showing the ratio between phosphorylated eNOS and total eNOS under basal conditions or after S1P stimulation. The data represent mean values ± SEM from three independent experiments.

3.4 S1P-stimulated activation of the small GTPase Rac in endothelial cells

3.4.1 S1P-mediated Rac activation in HUVEC

The Rho family small GTPases such as RhoA, Cdc42 and, in particular, Rac-1 are essential in regulation of actin cytoskeletal organisation and cell movement. Recent

investigations have been focused on the interaction between PI3K/Akt and Rac signalling pathways in S1P-stimulated cells.

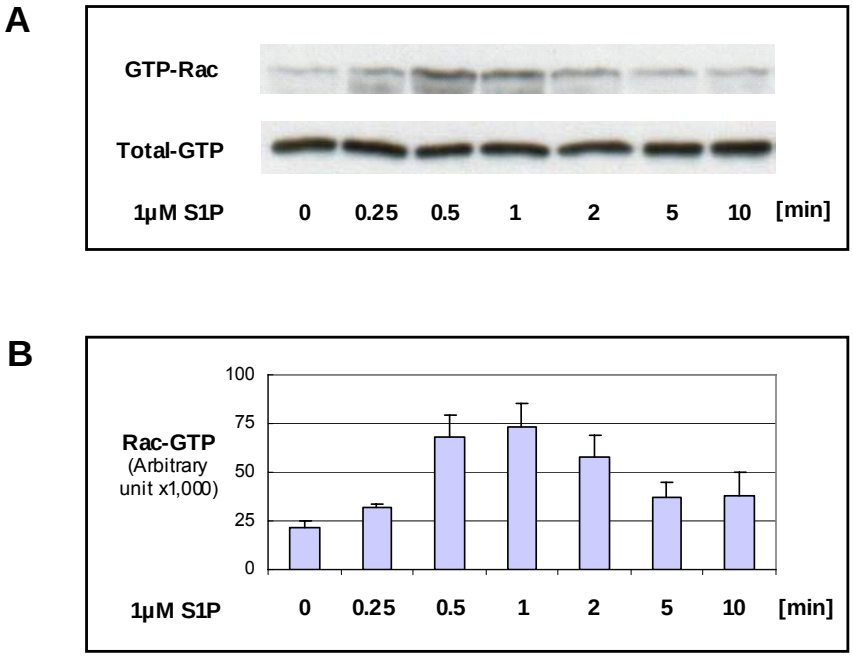

Figure 15. Time-dependency of S1P-induced Rac activation in HUVEC. HUVEC were plated on 60-mm dishes and Rac pull-down assays were performed when cells were confluent. Briefly, HUVEC were starved with M199/0.25 % HSA for 5 h and stimulated with 1 µM S1P. Cells were lysed with lysis buffer containing GST-PAK (20 µg/ml). Lysates were collected by centrifugation and the supernatant was further incubated with GSH-Sepharose beads for 30 min to pull-down GTP-loaded Rac. Samples were separated on SDS-PAGE and GTP-bound or total Rac were detected by immunoblotting using a specific antibody against Rac-1. **(A)**. Cells were stimulated with 1 µM S1P for different time points (0, 0.25, 0.5, 1, 2, 5, 10 min). Representative blots are shown. **(B)**. Densitometry analysis of Rac-GTP blots. The data represent mean values ± SD of two independent experiments.

In order to investigate the role of PI3K isoforms in S1P-induced Rac activation, we first characterised the effect of S1P on endogenous Rac-1 activity in HUVEC. The activation of Rac was measured in a pull-down assay as described above. As shown in Fig. 15, stimulation of HUVEC with 1 µM S1P triggered a rapid and potent activation

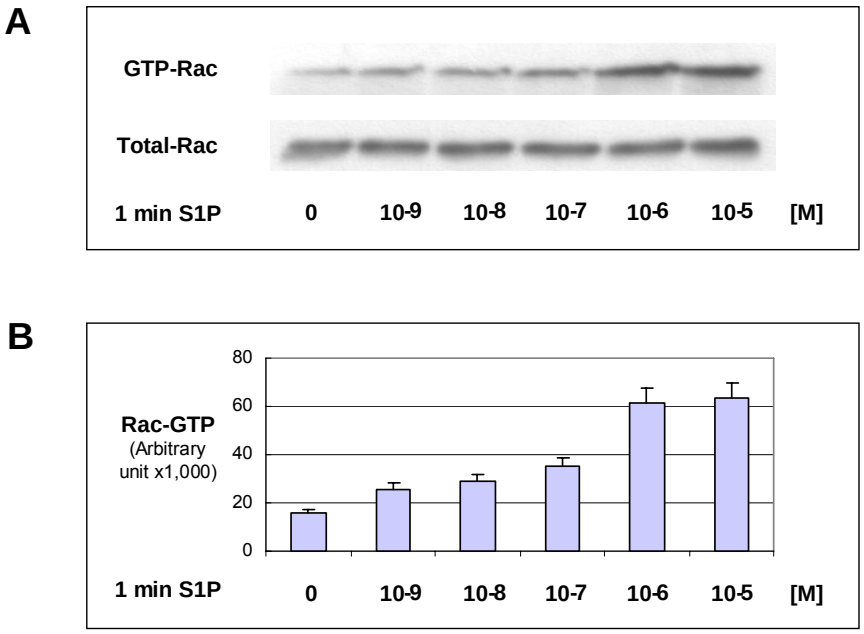

Figure 16. Concentration-dependency of S1P-induced Rac activation in HUVEC. Cells were plated on 60-mm dishes and were starved with M199/0.25 % HSA for 5 h. After S1P stimulation, cells were lysed with lysis buffer containing GST-PAK (20 µg/ml). Lysates were collected by centrifugation and the supernatant was further incubated with GSH-Sepharose beads for 30 min to pull-down GTP-bound Rac..Samples were separated on SDS-PAGE. GTP-bound or total Rac were detected by immunoblotting using a specific antibody against Rac-1. **(A)**. Different concentration (10^{-9} - 10^{-5} M) of S1P were applied to cells for 1 min. Representative blots are shown. **(B)**. Densitometry analysis of Rac-GTP blots. The data represent mean values ± SD of two independent experiments.

of Rac in a time-dependent manner. GTP-bound Rac was detectable after 15 sec and sustained up to 10 min with a peak observed at 1 min.

In addition, Rac activation is dependent on S1P concentration. We observed a slight activation of Rac with nanomolar concentrations of S1P, but 1 µM and 10 µM were required to achieve a significant response (Fig. 16). Thus, 1 µM S1P stimulation for 1 min was used as a standard condition for further GTP-Rac pull-down experiments.

3.4.2 Effect of PI3K inhibitors on S1P-mediated Rac activation in HUVEC

To examine whether PI3Kβ and/or PI3Kγ isoforms are involved in mediating Rac activation in HUVEC, cells were pretreated with the PI3Kβ-specific inhibitor TGX- 221 (100 nM, 30 min) or the PI3Kγ-specific inhibitor AS-252424 (1 μM, 30 min) before adding 1 μM S1P to the dishes. S1P-stimulated GTP-bound Rac was diminished by both inhibitors at a similar level (TGX-221: 52%, AS-252424: 46%), whereas basal levels of GTP-Rac were unaltered. This result implies that both isoforms are necessary, at least in part, for S1P-induced Rac activation (Fig 17).

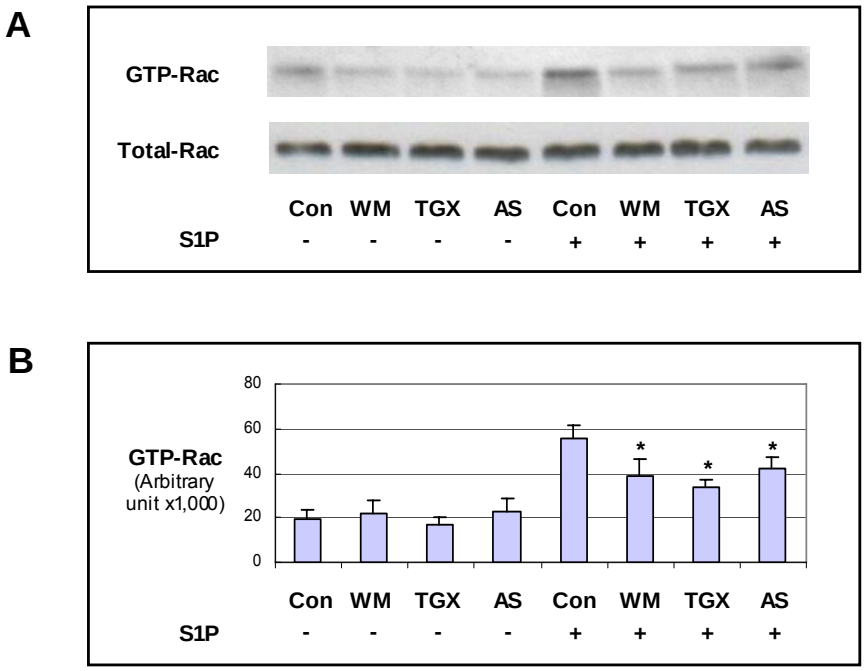

Figure 17. Effect of PI3K inhibitors on S1P-induced Rac activation in HUVEC. HUVEC were serum starved for 5 h and inhibitors (100 nM wortmannin (WM), 100 nM TGX-221 (TGX) and 1 μM AS-252424 (AS)) were applied during the last 30 min. Cells were subsequently stimulated with 1 μM S1P for 1 min and lysed with lysis buffer containing GST-PAK domain (20 μg/ml). Supernatant was incubated with GSH-Sepharose beads for 30 min to pull-down GTP-bound Rac. Immunoblotting analysis was performed using specific antibody against Rac1. **(A)**. One of five similar blots is shown. **(B)**. Densitometry analysis of Rac-GTP blots is shown. The data represent the mean values ± SEM derived from five independent experiments. *$p < 0.05$ versus S1P-stimulated control. Con, control.

3.4.3 Effect of PI3K inhibitors on S1P-mediated Rac activation in wild type and PI3Kγ knockout MLEC

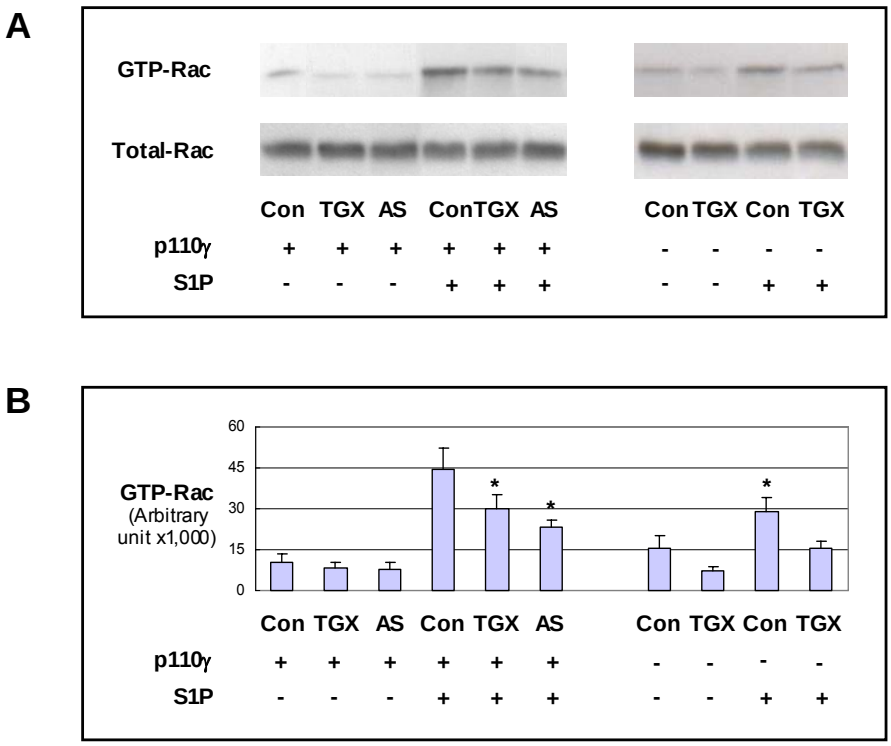

Figure 18. Effect of PI3K inhibitors on S1P-induced Rac activation in MLEC. Wild type and p110$\gamma^{-/-}$ MLEC were serum starved for 5 h and inhibitors (100 nM TGX-221 (TGX) or 1 µM AS-252424 (AS), 30 min) were applied during the last 30 min. Cells were subsequently stimulated with 1 µM S1P for 1 min and lysed with lysis buffer containing GST-PAK domain (20 µg/ml). Supernatant was incubated with GSH-Sepharose beads for 30 min to pull-down GTP-bound Rac. Immunoblotting analysis was performed using a specific antibody against Rac1. **(A)**. One of five similar blots is shown. **(B)**. Densitometry analysis of the Rac-GTP-blots is shown. The data show mean values ± SEM derived from five representative experiments. *$p < 0.05$ versus S1P-stimulated control in wild type MLEC. Con, control;

We also performed studies with specific PI3K inhibitors in murine endothelial cells (Fig. 18). In response to S1P stimulation (1 µM, 1 min), Rac activation was approximately 4-fold increased in wild type MLEC. When these cells were pretreated with 100 nM TGX-221 or 1 µM AS-252424, 30 min, S1P-induced activation of Rac was notably decreased (47% or 52%, respectively). Moreover, compared to wild type MLEC,

p110γ-negative MLEC displayed a 57% decrease of S1P-stimulated Rac activation. In addition, pretreatment of p110$\gamma^{-/-}$ cells with TGX-221 (100 nM, 30 min) was able to further downregulate Rac activation by about 50%.

3.5 S1P-induced migration in endothelial cells

3.5.1 S1P-mediated migration in HUVEC and MLEC

S1P has been demonstrated to be a potent stimulus of endothelial cell motility. Before investigating the role of PI3K isoforms in S1P-induced migration, we characterised the effect of S1P on migration of HUVEC and MLEC under our experimental conditions. The *in vitro* migration assay was performed using the "Transwell insert" system.
Previous data from our group have shown that S1P induced HUVEC migration in a time (2 and 4 h)- and concentration (0.01 – 10 µM)-dependent manner (data not shown). Furthermore, the migratory response to S1P was detected in MLEC. As shown in Fig. 19, MLEC revealed a significantly increased migratory response towards S1P (1 µM, 4 h).

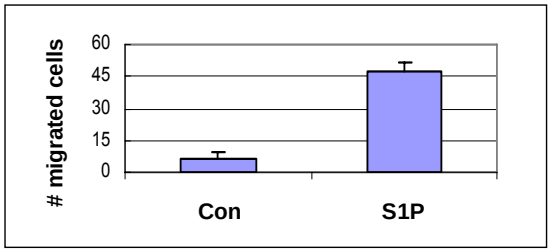

Figure 19. S1P-induced directional migration of wild type MLEC. 2.5 x 10^5 cells were seeded onto tissue culture insert. S1P was applied to the lower chamber and cells were allowed to migrate. Inserts were stained with hematoxylin and the number of migrated cell was counted. Migration was measured after stimulation of MLEC with S1P (1 µM, 4 h). Data are given as mean values ± SD (n=2). Con, control

3.5.2 Effect of PI3K inhibitors on S1P-mediated migration

Several studies have reported that S1P induces cell motility via a G_i protein-dependent pathway and the response was markedly reduced by PTX. Previous studies from our group confirmed these findings (data not shown). Moreover,

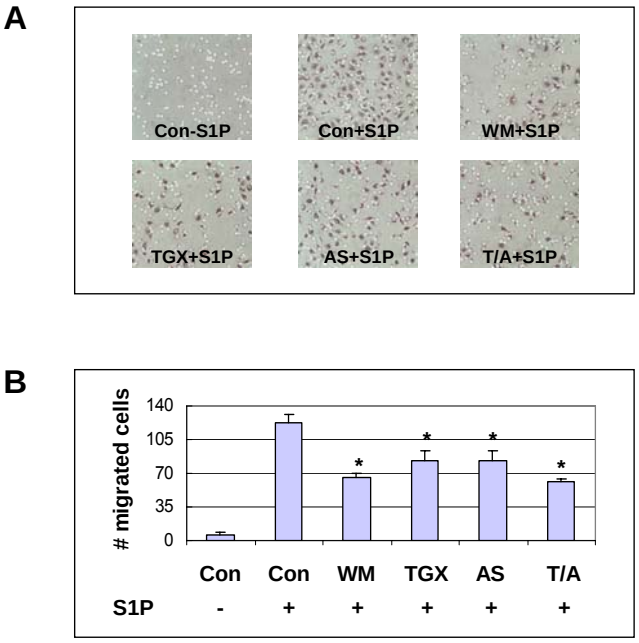

Figure 20. Effect of PI3K inhibitors on S1P-mediated migration. 2.5 x 10^5 HUVEC were seeded onto tissue culture insert in serum free medium. Cells were preincubated with 100 nM wortmannin (WM), 100 nM TGX-221 (TGX), 1µM AS-252424 (AS) and the combination of TGX and AS (T/A) for 30 min, respectively. Inhibitors were added to both upper and lower chambers, whereas 1 µM S1P was applied only to the lower chamber. Cells were allowed to migrate for 4 h. Thereafter, inserts were stained with hematoxylin and the number of migrated cell was counted. **(A)**. Representative microscopic images of cells migrating in the presence of S1P with or without inhibitor treatment are shown. **(B)**.The figure shows mean values ± SEM from four independent experiments. *p < 0.05 versus S1P-stimulated control. Con, control.

the involvement of PI3Ks in S1P-induced cell motility has been shown and accordingly, the non-selective PI3K inhibitor wortmannin (100 nM, 30 min) caused a significant reduction of S1P-triggered cell migration (47 %) in our study (Fig. 20).

Although it has been demonstrated that S1P-induced endothelial migration depends

on PI3K activation, the nature of PI3K isoforms involved has not yet been identified. Both PI3K isoforms, PI3Kβ and PI3Kγ, may contribute to cell migration via Gi-dependent pathways. In particular, PI3Kγ has been shown to be required for the migration of murine monocytes and neutrophils in response to chemotactic stimuli (Hirsch et al., 2000)

In the present study, we employed the inhibitors, TGX-221 and AS-252424, in transwell migration assays. As shown in Fig. 20, treatment of HUVEC with either 100 nM TGX-221 or 1 µM AS-252424 decreased migration by 32 % and 33 %, respectively. The combination of two inhibitors led to a reduction of 50 %, which was comparable to the level of inhibition achieved by wortmannin (47 %). These results illustrate that both PI3Kβ and PI3Kγ are important for directional cellular motility towards an S1P gradient.

3.5.3 Effect of PI3Kβ and γ overexpression on S1P-induced migration

In a second series of experiments, we overexpressed cDNA plasmids encoding a catalytically inactive mutant of PI3Kβ (p110β KR) or PI3Kγ (p110γ KR) in HUVEC, respectively. Transfections were carried out using the AMAXA Biosystems nucleofection procedure. The empty vector pcDNA3 was used as a control. Cells were transfected with 2 µg pcDNA3, p110β KR and p110γ KR plasmids, respectively. The level of the overexpressed proteins was detected in immunocomplexes (PI3Kβ) or protein lysates (PI3Kγ) by Western blotting (Fig. 21). Compared to pcDNA3 control, overexpression of p110β KR mutant inhibited the directional cell movement towards 1 µM S1P by 43 % (Fig. 21A). Similarly, overexpression of p110γ KR mutant led to a 40 % reduction of S1P-induced migration (Fig. 21B).

Taken together, our findings implicate that both PI3Kβ and PI3Kγ are involved in the regulation of S1P-induced chemotaxis of HUVEC.

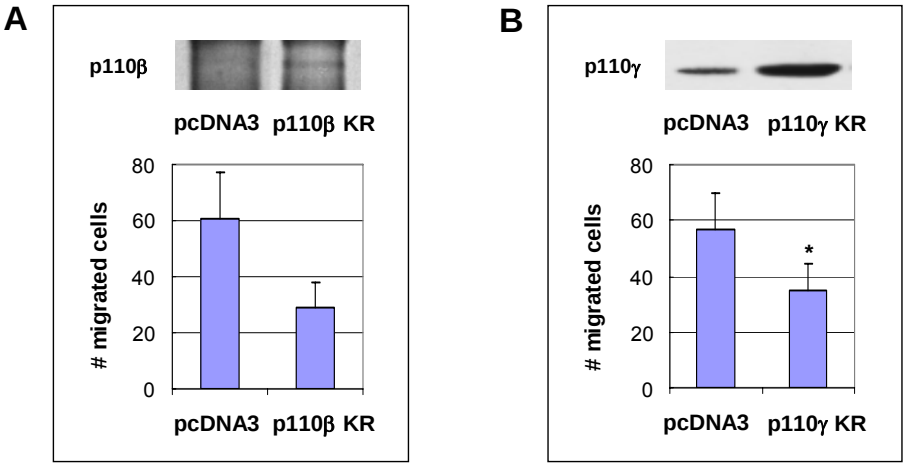

Figure 21. Effect of overexpression of p110ß KR or p110γ KR mutants on S1P-mediated migration. 1.0 x 10^6 HUVEC were transfected with 2 µg of p110ß KR (**A)** or p110γ KR **(B)** mutant cDNA plasmids, respectively, using the AMAXA Biosystems nucleofection procedure. 24 h post-transfection, 2,5 x 10^5 cells were detached and seeded onto tissue culture inserts in serum free medium.1 µM S1P was applied to the lower chamber. Cells were allowed to migrate for 4 h. Thereafter, inserts were stained with hematoxylin and the number of migrated cell was counted. An empty vector pcDNA3 was used as control. Data are given as mean values ± SEM (n=4 for p110ß KR mutant overexpression and n=5 for p110γ KR mutant experiments. Overexpression of mutants was detected in immunocomplexes (PI3Kβ) or protein lysates (PI3Kγ) by Western blotting with specific antibodies against PI3Kβ or PI3Kγ. *p < 0.05 versus S1P-stimulated pcDNA-transfected control.

3.5.4 Effect of PI3Kγ knockout on S1P-mediated migration in murine cells

To further explore the role of PI3Kγ in S1P-mediated endothelial migration, wild type and p110$\gamma^{-/-}$ MLEC were allowed to migrate towards 1 µM S1P for 4 h as described. S1P was able to stimulate murine endothelial cell migration in wild type and p110$\gamma^{-/-}$ MLEC. However, the absence of PI3Kγ catalytic subunit in these cells resulted in 48 % inhibition of cellular motility compared to wild type control (Fig. 22). To investigate whether PI3Kβ has an additional effect on migration in p110γ knockout cells, we pretreated normal and p110$\gamma^{-/-}$ MLEC with 100 nM TGX-221 (Fig. 22). Employment of the inhibitor significantly attenuated migration in wild type (51 ± 9 %) and also in p110γ

knockout cells (55 ± 9.8 %). On the contrary, 1µM AS-252424 had no effect on migration of p110γ-null MLEC any longer (data not shown). These results confirm our data from inhibitor study in HUVEC showing that TGX-221 and AS-252424 impaired cell migration at a similar extent. In conclusion, our results suggest that PI3Kβ and PI3Kγ contribute equally to S1P-stimulated chemotaxis in human and murine endothelial cells.

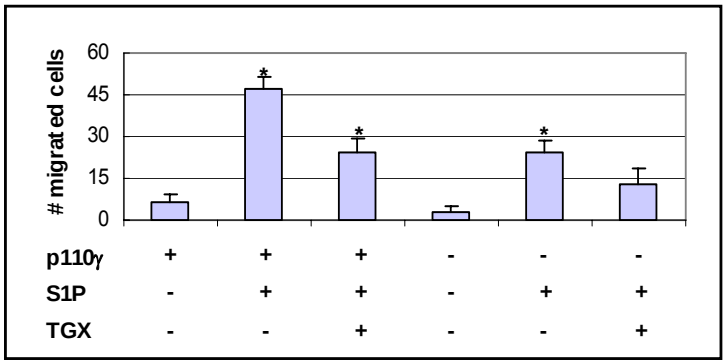

Figure 22. S1P-mediated migration in wild type and p110γ knockout MLEC. Cells (1.0 x 10^5) were seeded onto tissue culture inserts in serum free medium. 100nM TGX-221 was added to both upper and lower chamber for 30 min, 1 µM S1P was additionally applied to the lower chamber and a 4 h migration assay was performed. Data are given as mean values ± SEM (n=3). *p < 0.05 versus S1P-stimulated control.

3.5.5 Effect of eNOS inhibitor (L-NAME) on S1P-mediated migration

eNOS has been described to be an essential mediator in the regulation of endothelial cell migration in response to growth factors. However, studies on the role of eNOS in S1P-stimulated cell migration showed inconsistent results. To examine the function of eNOS in endothelial cell migration under our experimental conditions HUVEC were pretreated with 1 mM NOS inhibitor L-NAME for 30 min and consequently stimulated with 1 µM S1P for 4 h. As shown in Fig 23, L-NAME was unable to inhibit transwell cell migration towards S1P, suggesting the dispensability of eNOS activation in this reaction.

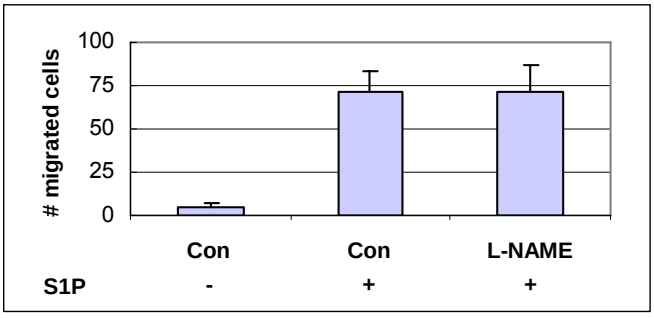

Figure 23. Effect of eNOS inhibitor on S1P-mediated migration. HUVEC (1.0 x 10^5) were seeded onto tissue culture insert in serum free medium. Cells were subsequently preincubated with 1 mM L-NAME for 30 min and then allowed to migrate towards S1P (1 µM) for 4 h. Thereafter, inserts were stained with hematoxylin and the number of migrated cell was counted. The figure shows mean values ± SEM from five independent measurements.

3.6 S1P-induced wound healing in endothelial cells

3.6.1 Effect of PI3K inhibitors on S1P-mediated wound healing in HUVEC

In contrast to transwell migration, which measures the movement of cells along a chemotactic gradient, the wound healing assay indicates the capability of cell movement induced by a constant dose of a stimulus. The mechanisms of regulating cell chemotaxis may differ from the control of chemokinesis and interestingly, PI3K isoforms probably have distinct functions in these processes. For instance, PI3Kγ has been shown to regulate neutrophil chemotaxis primarily by controlling the direction of cell migration (Hannigan et al., 2001). Moreover, it has also been indicated that overexpression of dominant negative mutant of p85 regulatory subunit reduced S1P-stimulated wound repair in BAEC (Rikitake et al., 2002). Thus, it becomes interesting to investigate the role of PI3K isoforms in a wound healing assay.

Therefore, HUVEC were pretreated for 30 min with 100 nM TGX-221 and 1 µM AS-252424. 1 µM S1P was applied and cells were allowed to move to the wound

region for 20 h. The number of migrated cells is shown in Fig. 24. S1P stimulated an approximately 2-fold increase of migration in HUVEC wound healing assays, which was significantly diminished by TGX-221. On the contrary, no effect of AS-252424 was detected in the same experiments. Both inhibitors did not alter the basal level of wound healing.

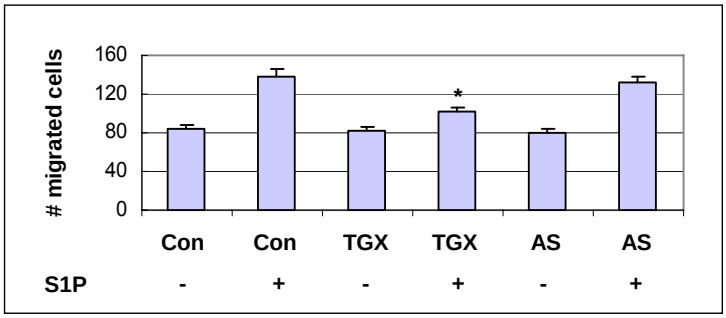

Figure 24. Effect of PI3K inhibitors on S1P-mediated wound healing in HUVEC. HUVEC were cultivated in 6-well plates and a wound area was scratched in the confluent monolayer. 100 nM TGX-221 or 1 µM AS-252424 was applied for 30 min. Cells were subsequently stimulated with 1 µM S1P for 20 h. The number of migrated cells was evaluated under microscope. The figure shows mean values ± SEM from four independent experiments. *p < 0.05 versus S1P-stimulated control.

3.6.2 Effect of PI3Kβ and PI3Kγ overexpression on S1P-induced wound healing

To further confirm our results, we overexpressed inactive mutants of p110β and p110γ, respectively, to investigate whether these mutants downregulate S1P-mediated migration in wound healing assays. HUVEC were transfected with 2 µg of control empty vector pcDNA3, p110β KR and p110γ KR mutant, respectively, using the AMAXA Biosystems nucleofection procedure. The transfected cell monolayers were scratched and stimulated with 1 µM S1P for 20 h. Number of cells moved into wound region was quantified and the result is shown in Fig. 25.

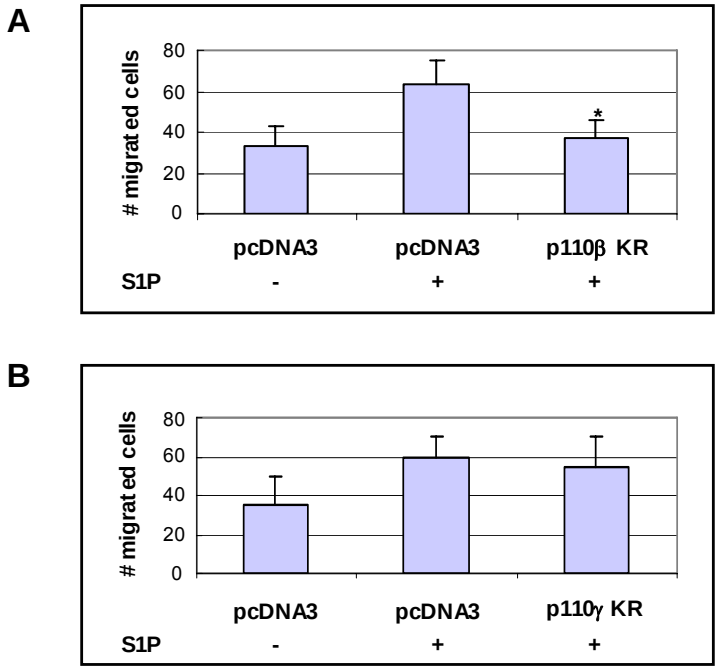

Figure 25. Effect of overexpression of p110ß KR and p110γ KR mutant on S1P-mediated wound healing. 1.0 x 10^6 HUVEC were transfected with 2 µg of p110ß KR **(A)** or p110γ KR **(B)** mutant cDNA plasmids, respectively, using the AMAXA Biosystems nucleofection procedure. Empty vector pcDNA3 was used as a control. 24 h post-transfection a wound region was scratched in the cell monolayer. Cells were stimulated with 1 µM S1P and the number of cells migrated into the wound was evaluated under microscope. The figure shows the mean values ± SEM from five independent experiments. *p < 0.05 versus S1P-stimulated control.

In agreement with our inhibitor studies, S1P-increased cell movement was significantly reduced by p110β KR overexpression, whereas transfection of p110γ KR mutant had no effect. These results reveal the essential role of PI3Kβ in mediating endothelial cell motility in response to a constant dose of S1P and suggest that PI3Kβ may be important during wound repair. In contrast, PI3Kγ was not required for S1P-stimulated migration in wound healing assay, although it was necessary to mediate endothelial cell chemotaxis.

4. Discussion

4. 1 Expression of PI3K in endothelial cells

4.1.1 Expression of PI3K catalytic subunit in endothelial cells

PI3Kβ and PI3Kγ have been demonstrated to be activated by G_i protein. The expression of PI3Kβ has been widely detected in mammalian cells, whereas PI3Kγ has been thought to be primarily expressed in leukocytes. Consequently, previous investigations of PI3Kγ function have been restricted to haematopoietic cells. During our current work, however, one group demonstrated that p110γ also exists in HUVEC and the END3.1b endothelial cell line and is required for neutrophil capturing and rolling by regulating selectin expression (Puri et al., 2005). Additionally, Frey et al. have shown more recently that p110γ is expressed in MLEC and regulates NADPH oxidase-dependent oxidant production (Frey et al., 2006).

Our data proved the existence of p110β and γ isoforms at the protein level in HUVEC and in primary MLEC and revealed a new functional role of both isoforms in endothelial migration (see below). Previous investigations in p110γ knockout animals have shown the importance of PI3Kγ in many cell responses. For example, $p110\gamma^{-/-}$ deficiency resulted in a lower level of directed migration in neutrophils and mast cells (Hirsch et al., 2000; Sasaki et al., 2000). Furthermore, the superoxide generation was defective in $p110\gamma^{-/-}$ neutrophils which were stimulated by the GPCR-coupled chemokine (Li et al., 2000). In these studies, an essential role of p110γ was demonstrated that was unable to be substituted by other PI3K isoforms although p110β may also be activated by G_i protein. In agreement, we observed differences in endothelial cell functional behaviour between wild type and PI3Kγ knockout MLEC although a similar protein level of p110β was found in both cell types. Our data also indicate that the lack of p110γ does not alter the expression of p110β in MLEC. The same result was found in $PI3K\gamma^{-/-}$ neutrophils, macrophages and splenocytes which

exhibited the same protein level of the PI3Kβ isoform as wild type cells (Hirsch et al., 2000).

4.1.2 Expression of PI3Kγ regulatory subunit in endothelial cells

Different from PI3K class IA, class IB PI3Kγ has been demonstated to associate with a 101 kDa adaptor protein, p101. To date, endogenous p101 has been found in U937 cells (a human monocyte lymphoma cell line), HepG2 cells (a hepatoma cell line), VSMC and leukocytes (Stephens et al., 1997; Metjian et al., 1999; Kaplan-Albuquerque et al., 2003; Suire et al., 2005). These findings, together with the fact that PI3Kγ regulates endothelial cell response, led us to explore whether this p101 subunit exists in endothelial cells. Interestingly, no p101 messenger RNA was observed in HUVEC using RT-PCR technique, although these cells express p110γ at both mRNA and protein level. In contrast, we detected p101 mRNA in either wild type or p110γ knockout mouse endothelial cells.

Initially, p101 has been termed as an “adaptor” protein, since it was able to associate with and regulate the binding of p110γ and Gβγ subunits in response to G-protein coupled receptors (Stephens et al., 1997; Krugmann et al., 1999). Furthermore, studies from our group illustrated the requirement of p101 for the PI3Kγ-dependent activation of JNK (Lopez-Ilasaca et al., 1998), while Maier has pointed out that p101 influences the lipid substrate preference of PI3Kγ and its binding protects p110γ from degradation (Maier et al., 1999). Nevertheless, some groups have detected PI3Kγ activation in the absence of p101, rendering the role of this adaptor protein is still controversial. In agreement, our data show that the p110γ catalytic subunit is functional and physiologically important in human endothelial cells without the assistance of the p101 adaptor protein. The variations between these findings probably result from difference in cell types. Additionally, the lack of p101 expression in human endothelial cells may point to other adaptor/regulatory subunits interacting with p110γ in human endothelial cells.

More recently, another non-catalytic subunit of p110γ, p87, was discovered and proved to bind to both p110γ and Gβγ subunits and to mediate PI3Kγ activation (Voigt et al., 2006). This report prompted us to hypothesise that p87 in HUVEC, instead of p101, regulates the function of PI3Kγ. Surprisingly, HUVEC did not display the expression of p87 mRNA. Thus, in HUVEC the PI3Kγ catalytic subunit is able to exist and function in the absence of p101 or p87 regulatory subunits. Unlike human endothelial cells, MLEC isolated from both wild type and p110γ knockout mice revealed the expression of both p101 and p87 mRNA. Interestingly, the expression level of both adaptors was not affected by the absence or presence of PI3Kγ. In contrast, Voigt et al. have demonstrated that in the absence of p110γ, p101 showed a lower stability than p87 (Voigt et al., 2006), whereas a reduction of both p101 and p87 expression was observed in p110γ-negative neutrophils (Suire et al., 2005). The presence of p101 and p87 in the same cell has been shown before (Suire et al 2005) suggesting that they contribute to different properties of p110γ protein. However, more studies are required to understand the function of these adaptors in murine endothelial cells.

In conclusion, our findings suggest that p101 or p87 is dispensable for the function of p110γ catalytic subunit in human endothelial cells, since p110γ activation was detected in HUVEC lacking the expressions of both regulatory subunits. On the other hand, MLEC express both adaptors and their impact on p110γ function should be further clarified.

4. 2 PI3K-dependent protein phosphorylation/activation in endothelial cells

4.2.1 S1P-stimulated Akt phosphorylation and its involvement in endothelial migration

Akt, a critical downstream substrate of PI3Ks, has been shown to control abundant cellular responses. Upon extracellular stimulation, Akt translocates to the plasma membrane via binding to $PI_{(3,4,5)}P_3$, where it becomes activated. S1P has previously been demonstrated to trigger this process. In agreement, our studies also show that stimulation of HUVEC with S1P results in a rapid and reversible increase of Akt phosphorylation. Furthermore, we confirm that G_i protein and PI3K activity are involved in S1P-induced Akt activation, since pretreatment of cells with the G_i protein inhibitor PTX or the PI3K inhibitor wortmannin completely inhibited Akt phosphorylation.

Gi protein has been shown to activate both PI3Kβ and PI3Kγ via its $\beta\gamma$ subunits, raising the hypothesis that both is forms are able to mediate Akt phosphorylation in S1P-stimulated cells. However, our data indicate that only PI3Kβ is responsible for S1P-induced Akt phosphorylation, since TGX-221, the PI3Kβ-specific inhibitor, was able to abolish S1P-induced Akt phosphorylation, whereas the PI3Kγ inhibitor AS-252424 had no effect. In addition, Akt phosphorylation was unaltered in p110$\gamma^{-/-}$ MLEC. Thus, we show here for the first time that S1P stimulates Akt phosphorylation in endothelial cells in a G_i protein-dependent and PI3Kβ-specific manner.

Consistent with our findings, Igarashi et al. have previously reported that S1P leads to a rapid and isoform-specific activation of immonoprecipitated PI3Kβ in BAEC that do not express PI3Kγ. Furthermore, S1P-triggered Akt phosphorylation was abolished by wortmannin in this cell type (Igarashi et al., 2001a; Igarashi and Michel, 2001b). Our results are in agreement with previous reports showing that the expression of PI3Kβ in murine fibroblasts is necessary and sufficient to transmit signals from G-protein

coupled receptors to Akt (Murga 2000). However, although our investigations suggest that S1P activates Akt in HUVEC and MLEC solely through a G_i/PI3Kβ-dependent pathway, the underlying mechanism is still unknown. Full Akt activity depends on its translocation from cytosol to the plasma membrane where it becomes phosphorylated at its amino acid residues threonine 308 and serine 473. This process may not only be dependent on binding of Akt to $PI_{(3,4,5)}P_3$ but may additionally require the p85 regulatory subunit which is binding only to PI3Kβ but not to PI3Kγ. However, further studies are needed to clarify a possible role of the p85 subunit.

Akt has been demonstrated to be a key effector to regulate cell migration. It has been reported that Akt translocates to the plasma membrane and colocalises with F-actin at the leading edge of cells in response to a chemoattractant gradient (Servant et al., 2000; Hannigan et al., 2002). To understand the role of PI3Kβ-mediated Akt activation in S1P-induced cell migration, we investigated the effect of TGX-221 which completely abolished phosphorylation of Akt. We found that TGX-221-treated cells show a 30 % to 40 % inhibited capability to migrate to an S1P gradient or to respond in wound healing assays suggesting that the PI3Kβ/Akt-dependent signalling pathway may play a role in S1P-stimulated cell motility in HUVEC and other pathways may exist.

In agreement with our studies, it has been revealed that overexpression of a dominant negative Akt mutant markedly inhibited S1P-mediated migration in Chinese hamster ovary (CHO) –K1 cells transfected with S1P receptor $S1P_1$, whereas this Akt mutant had no effect on migration of CHO cells which were transfected with another S1P receptor, $S1P_3$ (Lee et al., 2001). On the other hand, $S1P_1$-independent pathways exist in endothelial cells which may not signal via Akt. Inoki et al. have mentioned that S1P-stimulated migration was $S1P_3$/G_i-dependent in a murine endothelial cell line, SVEC4-10, which expresses $S1P_2$ and $S1P_3$, but not $S1P_1$ (Inoki et al., 2006). This may explain the wortmannin and TGX-221-insensitive part of S1P-stimulated cell movement. Moreover, S1P-induced Akt activation through $S1P_1$/G_i/PI3Kβ may not only be involved in cell motility, but also in other cellular responses, for instance in the

regulation of endothelial barrier integrity (Lee et al., 2006). Thus, we propose that the $S1P_1/G_i/PI3K\beta$ signalling pathway is important for S1P-stimulated Akt activation and that Akt activity is essentially but not solely responsible for endothelial cell motility upon S1P stimulation.

4.2.2 S1P-stimulated eNOS phosphorylation and its involvement in endothelial migration

eNOS is known to be a downstream effector of Akt and contributes to endothelial cell migration in response to growth factors. In the present study, we show that S1P promoted eNOS phosphorylation at the serine 1177 residue in both HUVEC and MLEC. Our data reveal that Akt/PI3Kβ is involved in this process since eNOS phosphorylation was inhibited by TGX-221 but not by the PI3Kγ-specific inhibitor AS-252424. Moreover, our studies in murine endothelial cells display that S1P is able to phosphorylate eNOS in wild type as well as in $p110\gamma^{-/-}$ cells and that the absence of this enzyme did not decrease S1P-induced eNOS phosphorylation at serine 1177. Thus we conclude that PI3Kγ does not contribute to S1P-induced eNOS activation. Neither PI3K inhibition with wortmannin nor PI3Kβ inhibition with TGX-221, however, was able to inhibit eNOS phosphorylation completely although Akt phosphorylation was blocked under these conditions suggesting that other signalling pathways were involved.

Previous observations have demonstrated that wound healing and angiogenesis was impaired in eNOS-deficient mice and that eNOS is required for proper endothelial cell migration, proliferation, and differentiation (Lee PC. et al., 1999). Interestingly, an involvement of NO in endothelial cells has mainly been attributed to growth factors (Dimmeler et al., 2000), whereas conflicting data exist concerning the role of NO in S1P-stimulated endothelial cell migration. Morales-Ruiz et al. have provided evidence that L-NAME inhibits VEGF-promoted but not S1P-stimulated migration in BAEC (Morales-Ruiz et al., 2001), while others showed that the eNOS inhibitor L-NAME

suppressed directional cell motility to an S1P gradient (Rikitake et al., 2002; Murohara et al., 1999), although the inhibition was lower than 20 %. In the present study, we failed to demonstrate an eNOS-dependent migration in response to S1P stimulation, since the pretreatment of cells with L-NAME was unable to affect cell movement.
Taken together, our data suppose that S1P-induced endothelial cell migration is independent from eNOS, although S1P is able to induce eNOS phosphorylation via G_i protein and PI3Kβ as well as via other pathways. Instead, eNOS may play a more important role in growth factor-regulated endothelial cell motility.

4.2.3 S1P-stimulated Rac activation

The Rho family small GTPase Rac regulates actin rearrangement and cytoskeleton remodeling such as the formation of ruffles and lamellipodia, thereby being a critical effector in cell migration. A previous investigation has shown that S1P stimulates Rac activation in endothelial cells, which in turn influences cell motility (Li et al., 2005). However, the underlying mechanism was not well characterised. In the present study, we demonstrated S1P-induced Rac1 activation in HUVEC and MLEC in a time- and concentration-dependent manner which was PTX-sensitive, suggesting the requirement of G_i protein. This is consistent with a previous study (Mehta et al., 2005). Similarly, the inhibition of the G_i protein-coupled receptor $S1P_1$ has been shown to attenuate S1P-induced Rac1 activation in HUVEC (Paik et al., 2001). PI3Ks are important downstream effectors of G_i protein and both PI3K and Rac have been shown to be involved in regulating S1P-induced endothelial cell migration. Accordingly, abrogation of PI3K by a general inhibitor, LY294002, reduced Rac1 activition upon S1P in human pulmonary artery endothelial cells (Singleton et al., 2005). In agreement, our findings illustrate that S1P-induced Rac1 activation is PI3K-dependent, since pretreatment of cells with wortmannin was able, at least in part, to inhibit the level of S1P-activated Rac. In contrast, another group failed to observe this response in BAEC (Gonzales et al., 2006), indicating that cell type specificity may

influence this process.

It has been demonstrated that stimulation of macrophages with the cytokines, RANTES (regulated on activation normal T cell expressed and secreted) or CSF-1 (colony-stimulating factor), which couple to GPCR or RTK, respectively, lead to a rapid and transient PI3K-dependent Rac activation (Weiss-Haljiti et al., 2004). This finding points to a possibility to activate Rac via different PI3K isoforms. Interestingly, our results demonstrate a significant decrease of S1P-stimulated Rac1 activation in HUVEC and wild type MLEC when cells were pretreated with either TGX-221 or AS-252424. Moreover, MLEC lacking p110γ showed a notable decrease of S1P-induced Rac activation, which was further downregulated by pretreatment with TGX-221. Thus, we suggest that both PI3Kβ and PI3Kγ are important for S1P-induced Rac activity.

S1P stimulates a wide range of cellular responses via the PI3K/Akt pathway. Several interactions exist between Akt and Rac1 which seem to be very complex and so far, incompletely understood. Akt has been demonstrated to be an upstream effector of Rac1 that plays both negative and positive roles. One group showed that Akt reduced Rac1 activity by phosphorylating Rac1 at the serine 71 residue and by inhibiting its GTP-binding activity in a human melanoma cell line, SK-MEL28 (Kwon et al., 2000). In contrast, Lee et al. have demonstrated that in CHO cells stably transfected with the $S1P_1$ cDNA sequence, Akt associates with and directly phosphorylates $S1P_1$ at threonine 236 upon S1P stimulation which in turn was responsible for Rac1 activation. Overexpression of a kinase dead mutant of Akt in these cells led to a full abrogation of S1P-mediated Rac1 activation (Lee et al., 2001). On the other hand, Akt was also suggested to be downstream of Rac1. The transfection of a constitutively active Rac1 markedly elicited Akt activity, while knockdown of Rac1 by a specific siRNA entirely abolished S1P-stimulated Akt phosphorylation (Genot et al., 2000; Gonzalez et al., 2006). Interestingly, the membrane translocation of Akt via its PH domain appears to be essential for Akt activation by active state Rac (Wang et al., 2002). In the current study, S1P-triggered Akt activation was completely inhibited by PI3K inhibitors

wortmannin and TGX-221, while these inhibitors only partially reduced Rac1 activity. Taken together, we suggest an involvement of Akt in S1P-induced Rac activation upon S1P/GPCR/PI3Kβ signalling pathway in endothelial cells, but Rac1 may be also activated by Akt-independent signalling molecules in response to S1P stimulation.

Rac activity was also diminished in HUVEC pretreated with a PI3Kγ-specific inhibitor or in p110$\gamma^{-/-}$ MLEC, whereas in both situations Akt phosphorylation was unaltered. This result illustrates that PI3Kγ-dependent Rac activation in response to S1P occurred in an Akt-independent manner. Therefore, the interesting question remains, through which signalling pathways PI3Kγ may regulate Rac activity. One possibility is the involvement of PI3Kγ in the regulation of GEFs such as Vav, Tiam, SWAP-70 and P-Rex families. It has been shown, for example, that expression of an inactive mutant of PI3Kγ blocked Rac-dependent actin rearrangement in fMLP-stimulated COS-7 cells (Ma et al., 1998). Furthermore, the generation of $PI_{(3,4,5)}P_3$ in response to PI3K activation enhanced Vav activity (Han et al., 1998). Recently, Li et al. have reported that co-expression of the GEF P-Rex2b with Gβγ subunits in HEK293 cells, increased Rac1 activation, which was further enhanced by co-expression of P-Rex2b, Gβγ and PI3Kγ or a constitutively active mutant of PI3K. Moreover, suppression of endogenous P-Rex2b by siRNA significantly inhibited Rac1 activation and cell migration in S1P-stimulated HUVEC (Li et al., 2005), indicating that P-Rex2b is partially required for S1P-induced Rac1 activation and endothelial migration via a Gβγ/PI3Kγ signalling pathway. Recently, another Rac-GEF SWAP70 has been demonstrated to bind specifically to $PI_{(3,4,5)}P_3$ and move to membrane ruffles upon growth factor stimulation (Shinohara et al., 2002). Furthermore, it was found to bind to activated Rac and colocalise with F-actin thereby regulating actin rearrangement. Accordingly, growth factor-induced Rac activity, membrane ruffle formation and cell migration were impaired in SWAP70$^{-/-}$ cells or in cells that expressing a dominant negative mutant of SWAP70 (Shinohara et al., 2002; Ihara et al., 2006; Sivalenka et al., 2004). Finally it should be noted that Tiam1 has been shown to be a possible candidate in S1P-mediated Rac activation (Singleton 2005, Gonzalez 2006).

In summary, our study demonstrates that both PI3Kβ and PI3Kγ are involved in S1P-induced Rac1 activation in HUVEC and MLEC. We suggest that these two isoforms may influence Rac activation through, at least in part, distinct signalling pathways. The individual effect of distinct PI3K isoforms may vary dependent on the different stimuli and cell types. Finally, it should also be noted that we observed a PTX-sensitive but PI3K-independent part of Rac activation, indicating that some additional G_i-mediated signalling pathways are involved in Rac activation in S1P-stimulated endothelial cells.

4. 3 PI3K-dependency of S1P-induced endothelial cell motility

4.3.1 S1P-stimulated directional endothelial cell migration

When cells are exposed to a chemoattractant agent, they exhibit the ability to move into the direction of the gradient. S1P has been demonstrated to be one of potent stimuli of cell motility in many cell types, including fibroblasts, smooth muscle cells, neutrophils and myoblasts. In most cases, PI3K activation has been found to be involved. The PI3K-regulated directional cell movement involves (1) the sensing of a chemokine, (2) the recruitment of PH domain-containing proteins at the plasma membrane via interaction with $PI_{(3,4,5)}P_3$ generated by PI3Ks, (3) the differential polymerisation of F-actin at the leading edge resulting in a defined cell polarity, (4) the new adhesion at the front of the cell and (5) the sequential contraction of the body and rear of the cell. Cell migration is a fundamental process that contributes to diverse biological responses. For example, the directed migration of polymorphonuclear leukocytes and macrophages is a critical process in inflammation (Devreotes and Zigmond, 1988; Downey et al., 1994). PI3Kγ seems to be dispensable for controlling the direction of cell movement. Indeed, it has been dubbed the compass of neutrophils (Rickert et al., 2000). Several groups have reported that the production of

$PI_{(3,4,5)}P_3$ and cell chemotaxis induced by chemokines such as IL-8, fMLP, and C5a were impaired in p110γ gene-deficient murine neutrophils (Sasaki et al., 2000; Li et al., 2000; Hannigan et al., 2002). Similar results have been found in $p110\gamma^{-/-}$ dendritic cells and macrophages as well as in human melanoma cells transfected with an inactive mutant of PI3Kγ (Prete et al., 2004; Hirsch et al., 2000; Lee et al., 2002). So far, it is not known, however, which PI3K isoforms mediate migration and whether PI3Kγ plays a similar role in endothelial cells as in neutrophils.

In the present study, we first explored the role of PI3K isoforms in S1P-induced cell motility in HUVEC and MLEC. Endothelial cells were exposed to an S1P gradient after pretreatment with PI3K isoform-specific inhibitors or transfection with kinase dead mutants of PI3Ks, respectively. Our results show for the first time that PI3Kγ is, at least in part, responsible for human and murine endothelial cell movement towards an S1P gradient since S1P-induced transwell migration was inhibited by the PI3Kγ-specific inhibitor AS-252424 or by overexpression of the p110γ KR mutant. This conclusion is further supported by showing impaired migration towards S1P in p110γ-deficient MLEC. In addition, our data show for the first time that PI3Kβ contributes to endothelial cell migration, since HUVEC pretreated with TGX-221 or transfected with the p110β KR mutant also revealed a decreased migratory response towards S1P. Moreover, TGX-221 was able to further inhibit migration in $p110\gamma^{-/-}$ MLEC. In agreement with these findings, one group has reported that microinjection of a PI3Kβ-specific antibody significantly reduced lamellipodia extension and cell migration in macrophages (Vanhaesebroeck et al., 1999b). Our data suggest that both PI3K isoforms are necessary to mediate endothelial cell migration in response to an S1P gradient, the underlying mechanism are, however, not well understood.

The product of PI3K lipid kinase activity, $PI_{(3,4,5)}P_3$, has been indicated to be an important player in the regulation of cell polarity by generating an intracellular gradient necessary for polarised signalling and cell movement. Our results suggest the possibility that both PI3Kβ and PI3Kγ activation in endothelial cells may be essential for S1P-mediated $PI_{(3,4,5)}P_3$ generation although in neutrophils PI3Kγ has been

demonstrated to be the major isoform responsible for fMLP-induced $PI_{(3,4,5)}P_3$ production (Wu et al., 2000). Recent studies have demonstrated the indispensability of PI3K class IA for chemokine-increased $PI_{(3,4,5)}P_3$ level (Boulven et al., 2006; Condliffe et al., 2005). On the other hand, different PI3K isoforms may have distinct effects on signalling molecules such as Akt or Rac that have been known to be essential regulators of cell motility. As discussed above, Akt translocates to the leading edge of cells in response to a chemoattractant to trigger cell migration (Parent et al., 1998; Servant et al., 2000), while cells lacking Akt are unable to properly polarise when placed into a chemotactic gradient (Chung and Firtel, 2002). In addition, the importance of Rac and Akt in regulating directed cell migration was confirmed by a significantly diminished formation of lamellipodia and cell motility in endothelial cells transfected with inactive mutants of Akt or Rac (Lee et al., 2001; Lee et al., 2006). Since S1P-stimulated Akt phosphorylation at serine 473 residue can be totally blocked by a PI3Kβ-specific inhibitor, we hypothesise that PI3Kβ may primarily transmit the signal via the downstream effector Akt further leading to Akt-dependent Rac activation or to activation of other Akt-dependent pathways. In addition, PI3Kβ may activate Rac-GEFs directly. PI3Kγ may probably mainly function through activation of the small GTPase Rac via GEFs and may be especially important for actin reorganisation at the leading edge (Fig. 26). It should also be noted, however, that S1P-induced migration was not completely reduced by wortmannin or the combination of PI3Kβ and PI3Kγ inhibitors, indicating the existence of other Gi protein-dependent signalling pathways.

4.3.2 S1P-stimulated endothelial cell migration in wound healing

When tissues are injured in living organisms, the endothelium contributes partially to wound healing by increasing proliferation and angiogenesis. S1P generated upon platelet activation may participate in wound healing as a mediator of this process. Therefore, the motility of HUVEC was further evaluated *in vitro* by a wound healing

assay. In contrast to migration towards a chemotactic gradient, the wound healing assay investigates cell movement in the presence of a constant dose of the chemoattractant. Previous studies have already shown that S1P increased motility in wound healing assays in HUVEC and BAEC (Lee et al., 2000).

Our results demonstrate that S1P-induced HUVEC wound healing was significantly reduced by impairment of PI3Kβ with either an isoform-specific inhibitor or a dominant negative mutant. On the contrary, inhibition of PI3Kγ showed no effect on S1P-induced cell movement into the wound region. Our data demonstrate for the first time that migration of endothelial cells in the absence of a chemoattractant gradient requires only PI3Kβ but not PI3Kγ activation. These data are in agreement with findings from fMLP-stimulated $p110\gamma^{-/-}$ neutrophils showing that only cell chemotaxis was decreased in these cells whereas chemokinesis was not impaired (Hannigan et al. 2001). In this study, it was demonstrated that the lack of directionality likely results from a defect in the ability to stabilise and consolidate a leading edge. The authors postulate that in fMLP-stimulated neutrophils the function of PI3Kγ is to produce $PI_{(3,4,5)}P_3$ at the leading edge which would recruit PH domain containing proteins to the cell membrane of the leading edge and in turn cause the redistribution of F-actin and other relevant proteins to provide the directionality for cell movement. Similar mechanisms could play a role in endothelial cells responding to a chemotactic gradient. In contrast, the role of PI3Kγ may be dispensable for the control of the speed of migration and PI3Kβ may have a major role in the regulation of random migration. In agreement with this hypothesis, the overexpression of a dominant negative mutant of p85, the regulatory subunit of PI3K class IA, resulted in a notable decrease of S1P-induced cell migration into the wounded area of a BAEC monolayer (Rikitake et al., 2002), pointing to a role of PI3Kβ in this process. Akt may be one of the PI3Kβ downstream effectors responsible for the regulation of chemokinesis, since cells lacking Akt move slowly (Chung and Firtel, 2002). Moreover, transfection of HUVEC or BAEC with dominant negative Akt significantly attenuated wound healing upon S1P stimulation (Lee et al., 2006; Rikitake et al., 2002). In addition, our data revealed

PI3Kβ as the only PI3K isoform signalling through Akt suggesting that Akt is involved in PI3Kβ-mediated random migration. Since PI3Kβ inhibition also diminished Rac activation, Rac may also contribute to cell migration in wound healing assays. Overexpression of an inactive form of Rac1 in neutrophils resulted in an inefficient "tail" retraction at the backward side of cells (Pestonjamasp et al., 2006).

In conclusion, our study suggests that a chemotactic S1P gradient is able to increase endothelial cell motility through G_i-mediated PI3K activation. Different isoforms, PI3Kβ and PI3Kγ, which are known to be activated by G_i protein βγ subunits, are essential to mediate the directional cell movement. On the contrary, our results show that only PI3Kβ is required for the S1P-mediated endothelial migration in wound healing assays. Moreover, S1P stimulation leads to Akt phosphorylation only via PI3Kβ, whereas S1P-induced Rac activation is dependent on both PI3Kβ and PI3Kγ activities.

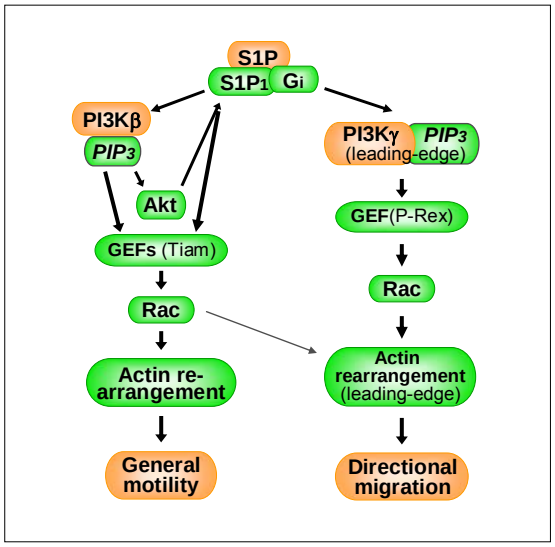

Figure 26. Model for the involvement of PI3Kβ and PI3Kγ isoforms in S1P-induced endothelial cell migration. The model suggests a hypothesis that S1P is able to stimulate endothelial cell migration through both PI3Kβ and PI3Kγ pathways. PI3Kβ may primarily transmit the signal via the downstream effector Akt further leading to Akt-dependent activation of small GTPase Rac. In addition, PI3Kβ may activate Rac-GEFs directly. PI3Kγ may mainly function through activation of Rac via GEFs and may be especially important for actin reorganisation at the leading edge. Furthermore, both PI3K isoforms may amplify each other via their downstream effectors.

5. References

- Ackah E, Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, Ouchi N, Easton RM, Galasso G, Birnbaum MJ, Walsh K, Sessa WC. (2005) Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *J clin Invest*. **115**, 2119-2127.
- Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P. (1996) Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett*. **399**, 333-338.
- Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med*. **1**, 1024-1028.
- Ancellin N, Colmont C, Su J, Li Q, Mittereder N, Chae SS, Stefansson S, Liau G, Hla T. (2002) Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. *J Biol Chem*. **277**, 6667-6675.
- Becciolini L, Meacci E, Donati C, Cencetti F, Rapizzi E, Bruni P. (2006) Sphingosine 1-phosphate inhibits cell migration in C2C12 myoblasts. *Biochim Biophys Acta.* **1761**, 43-51.
- Bellacosa A, Testa JR, Staal SP, Tsichlis PN. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*. **254**, 274-277.
- Bondev A, Rubio I, Wetzker R. (1999) Differential regulation of lipid and protein kinase activities of phosphoinositide 3-kinase gamma in vitro. *Biol Chem.* **380**, 1337-1340.
- Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R, Wymann MP. (1998) Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science.* **282**, 293-296.

- Boulven I, Levasseur S, Marois S, Pare G, Rollet-Labelle E, Naccache PH. (2006) Class IA phosphatidylinositide 3-kinases, rather than p110 gamma, regulate formyl-methionyl-leucyl-phenylalanine-stimulated chemotaxis and superoxide production in differentiated neutrophil-like PLB-985 cells. *J Immunol*. **176**, 7621-7627.
- Burridge K, Wennerberg K. (2004) Rho and Rac take center stage. *Cell*. **116**, 167-179.
- Carmeliet P. (2003) Angiogenesis in health and disease. *Nat Med*. **9**, 653-660.
- Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV. (2005) Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat Med*. **11**, 1188-1196.
- Condliffe AM, Davidson K, Anderson KE, Ellson CD, Crabbe T, Okkenhaug K, Vanhaesebroeck B, Turner M, Webb L, Wymann MP, Hirsch E, Ruckle T, Camps M, Rommel C, Jackson SP, Chilvers ER, Stephens LR, Hawkins PT. (2005) Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. *Blood*. **106**, 1432-1440.
- Coffer PJ, Woodgett JR. (1991) Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem*. **201**, 475-481.
- Czupalla C, Culo M, Muller EC, Brock C, Reusch HP, Spicher K, Krause E, Nurnberg B. (2003) Identification and characterization of the autophos-phorylation sites of phosphoinositide 3-kinase isoforms beta and gamma. *J Biol Chem.* **278**, 11536-11545.
- Galley HF, Webster NR. (2004) Physiology of the endothelium. *Br J Anaesth*. **93**, 105-113.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*. **91**, 231-241.

- Del Prete A, Vermi W, Dander E, Otero K, Barberis L, Luini W, Bernasconi S, Sironi M, Santoro A, Garlanda C, Facchetti F, Wymann MP, Vecchi A, Hirsch E, Mantovani A, Sozzani S. (2004) Defective dendritic cell migration and activation of adaptive immunity in PI3Kgamma-deficient mice. *EMBO J*. **23**, 3505-3515.
- Devreotes PN, Zigmond SH. 1988 Chemotaxis in eukaryotic cells: a focus on leukocytes and Dictyostelium. *Annu Rev Cell Biol*. **4**, 649-686.
- Dimmeler S, Dernbach E, Zeiher AM. (2000) Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration. *FEBS Lett*. **477**, 258-62.
- Domin J and Waterfield MD. (1997) Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS Lett*. **410**, 91-95.
- Downey GP. (1994) Mechanisms of leukocyte motility and chemotaxis. *Curr Opin Immunol*. **6**, 113-124.
- Endo A, Nagashima K, Kurose H, Mochizuki S, Matsuda M, Mochizuki N. (2002) Sphingosine 1-phosphate induces membrane ruffling and increases motility of human umbilical vein endothelial cells via vascular endothelial growth factor receptor and CrkII. *J Biol Chem*. **277**, 23747-23754.
- Fenteany G, Zhu S. (2003) Small-molecule inhibitors of actin dynamics and cell motility. *Curr Top Med Chem*. **3**, 593-616.
- Frey RS, Gao X, Javaid K, Siddiqui SS, Rahman A, Malik AB. Phosphatidylinositol 3-kinase gamma signaling through protein kinase Czeta induces NADPH oxidase-mediated oxidant generation and NF-kappaB activation in endothelial cells. *J Biol Chem*. **281**, 16128-16138.
- Genot EM, Arrieumerlou C, Ku G, Burgering BM, Weiss A, Kramer IM. (2000) The T-cell receptor regulates Akt (protein kinase B) via a pathway involving Rac1 and phosphatidylinositide 3-kinase. *Mol Cell Biol*. **20**, 5469-5478.
- Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev*. **13**, 1422-1437.

- Gonzalez E, Kou R, Michel T. (2006) Rac1 modulates sphingosine 1-phosphate-mediated activation of phosphoinositide 3-kinase/Akt signaling pathways in vascular endothelial cells. *J Biol Chem*. **281**, 3210-3216.
- Goueffic Y, Guilluy C, Guerin P, Patra P, Pacaud P, Loirand G. (2006) Hyaluronan induces vascular smooth muscle cell migration through RHAMM-mediated PI3K-dependent Rac activation. *Cardiovasc Res*. **72**, 339-348.
- Hall A. (2005) Rho GTPases and the control of cell behaviour. *Biochem Soc Trans*. **33**, 891-895.
- Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, Krishna UM, Falck JR, White MA, Broek D. (1998) Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science.* **279**, 558-560.
- Hannigan M, Zhan L, Li Z, Ai Y, Wu D, Huang CK. (2002) Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis*. Proc Natl Acad Sci.* **99**, 3603-3608.
- Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. (2001) p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. *Proc Natl Acad Sci*. **98**, 9666-9670
- Hazeki O, Okada T, Kurosu H, Takasuga S, Suzuki T, Katada T. (1998) Activation of PI 3-kinase by G protein betagamma subunits. *Life Sci*. **62**, 1555-1559.
- Hla T, Maciag T. (1990) An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem*. **265**, 9308-9313.
- Hiles ID, Otsu M, Volinia S, Fry MJ, Gout I, Dhand R, Panayotou G, Ruiz-Larrea F, Thompson A, Totty NF. (1992) Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell*. **70**, 419-429.

- Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP. (2000) Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*. **287**, 1049-1053.
- Hu P, Mondino A, Skolnik EY, Schlessinger J. (1993) Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. *Mol Cell Biol.* **13**, 7677-7688.
- Hughes SK, Wacker BK, Kaneda MM, Elbert DL. (2005) Fluid shear stress modulates cell migration induced by sphingosine 1-phosphate and vascular endothelial growth factor. *Ann Biomed Eng.* **33**, 1003-1014.
- Igarashi J, Bernier SG, Michel T. (2001a) Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J Biol Chem*. **276**, 12420-12426.
- Igarashi J and Michel T. (2001b) Sphingosine 1-phosphate and isoform-specific activation of phosphoinositide 3-kinase beta. Evidence for divergence and convergence of receptor-regulated endothelial nitric-oxide synthase signaling pathways. *J Biol Chem*. **276**, 36281-36288.
- Igarashi J, Erwin PA, Dantas AP, Chen H, Michel T. (2003) VEGF induces S1P1 receptors in endothelial cells: Implications for cross-talk between sphingolipid and growth factor receptors. *PNAS*. **100**, 10664-10669.
- Inoki I, Takuwa N, Sugimoto N, Yoshioka K, Takata S, Kaneko S, Takuwa Y. 2006 Negative regulation of endothelial morphogenesis and angiogenesis by S1P2 receptor. *Biochem Biophys Res Commun*. **346**, 293-300.
- Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, Natesan S, Brugge JS. (2005) Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. *J Cell Biol*. **171**, 1023-1034.
- Jimenez C, Hernandez C, Pimentel B, Carrera AC. (2002) The p85 regulatory subunit controls sequential activation of phosphoinositide 3-kinase by Tyr kinases and Ras. *J Biol Chem*. **277**, 41556-41562.

- Jones PF, Jakubowicz T, Pitossi FJ, Maurer F, Hemmings BA. (1991) Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc Natl Acad Sci.* **88**, 4171-4175.
- Kandel, E.S. and Hay, N. (1999) The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res*. **253**, 210-229.
- Kapeller R, Prasad K.V., Janssen O., Hou W., Schaffhausen B.S., Rudd C.E., Cantley L.C. (1994) Identification of two SH3-binding motifs in the regulatory subunit of phosphatidylinositol 3-kinase. *J Biol Chem.,* **269**, 1927-1933.
- Kaplan-Albuquerque N, Garat C, Desseva C, Jones PL, Nemenoff RA. (2003) Platelet-derived growth factor-BB-mediated activation of Akt suppresses smooth muscle-specific gene expression through inhibition of mitogen-activated protein kinase and redistribution of serum response factor. J Biol Chem. **278**, 39830-39838.
- Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. (2001) Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*. **17**, 615-675.
- Kim HS, Skurk C, Thomas SR, Bialik A, Suhara T, Kureishi Y, Birnbaum M, Keaney JF Jr, Walsh K. (2002) Regulation of angiogenesis by glycogen synthase kinase-3beta. *J Biol Chem*. **277**, 41888-41896.
- Kimura T, Watanabe T, Sato K, Kon J, Tomura H, Tamama K, Kuwabara A, Kanda T, Kobayashi I, Ohta H, Ui M, Okajima F. (2000) Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. *Biochem J.* **348**, 71-76.
- Kivens WJ, Hunt SW, Mobley JL, Zell T, Dell CL, Bierer BE, Shimizu Y. (1998) Identification of a proline-rich sequence in the CD2 cytoplasmic domain critical for regulation of integrin-mediated adhesion and activation of phosphoinositide 3-kinase. *Mol Cell Biol*. **18**, 5291-5307.
- Kluk MJ and Hla T. (2001) Role of the sphingosine 1-phosphate receptor EDG-1 in vascular smooth muscle cell proliferation and migration. *Circ Res*. **89**, 496-502.

- Krugmann S, Eguinoa A, McGregor AH, Hawkins PT, Stephens LR. (1997) Structural analysis of a novel isoform of phosphoinositide 3OH-kinase. *Biochem Soc Trans.* **25**, S604.
- Krugmann S, Hawkins PT, Pryer N, Braselmann S. (1999) Characterizing the interactions between the two subunits of the p101/p110gamma phosphoinositide 3-kinase and their role in the activation of this enzyme by G beta gamma subunits.*J Biol Chem*. **274**, 17152-17158.
- Kwon T, Kwon DY, Chun J, Kim JH, Kang SS. (2000) Akt protein kinase inhibits Rac1-GTP binding through phosphorylation at serine 71 of Rac1. *J Biol Chem.* **275**, 423-428.
- Lee H, Goetzl EJ, An S. (2000) Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am J Physiol Cell Physiol*. **278**, C612-C618.
- Lee HY, Bae GU, Jung ID, Lee JS, Kim YK, Noh SH, Stracke ML, Park CG, Lee HW, Han JW. (2002) Autotaxin promotes motility via G protein-coupled phosphoinositide 3-kinase gamma in human melanoma cells. *FEBS Lett*. **515**, 137-140.
- Lee JF, Ozaki H, Zhan X, Wang E, Hla T, Lee MJ. (2006a) Sphingosine 1-phosphate signaling regulates lamellipodia localization of cortactin complexes in endothelial cells. *Histochem Cell Biol*. **126**, 297-304.
- Lee JF, Zeng Q, Ozaki H, Wang L, Hand AR, Hla T, Wang E, Lee MJ. (2006b) Dual roles of tight junction-associated protein, zonula occludens-1, in sphingosine 1-phosphate-mediated endothelial chemotaxis and barrier integrity. *J Biol Chem*. **281**, 29190-29200.
- Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, Hla T. (1999) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell*. **99**, 301-312.

- Lee MJ, Thangada S, Paik JH, Sapkota GP, Ancellin N, Chae SS, Wu M, Morales-Ruiz M, Sessa WC, Alessi DR, Hla T. (2001) Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol Cell*. **8**, 693-704.
- Lee OH, Kim YM, Lee YM, Moon EJ, Lee DJ, Kim JH, Kim KW, Kwon YG. (1999) Sphingosine 1-phosphate induces angiogenesis: its angiogenic action and signaling mechanism in human umbilical vein endothelial cells. *Biochem Biophys Res Commun*. **264**, 743-750.
- Lee PC, Salyapongse AN, Bragdon GA, Shears LL 2nd, Watkins SC, Edington HD, Billiar TR. (1999) Impaired wound healing and angiogenesis in eNOS-deficient mice. *Am J Physiol*. **277**, H1600-1608.
- Leopoldt D, Hanck T, Exner T, Maier U, Wetzker R, Nurnberg B. (1998) Gbetagamma stimulates phosphoinositide 3-kinase-gamma by direct interaction with two domains of the catalytic p110 subunit. *J Biol Chem*. **273**, 7024-7029.
- Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV, Wu D. (2000) Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science*. **287**, 1046-1049.
- Li Z, Paik JH, Wang Z, Hla T, Wu D. (2005) Role of guanine nucleotide exchange factor P-Rex-2b in sphingosine 1-phosphate-induced Rac1 activation and cell migration in endothelial cells. *Prostaglandins Other Lipid Mediat*. **76**, 95-104.
- Liu X, Marengere LE, Koch CA, Pawson T. The v-Src SH3 domain binds phosphatidylinositol 3'-kinase. *Mol Cell Biol.* **13**, 5225-5232.
- Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest*. **106**, 951-961.
- Long JS, Natarajan V, Tigyi G, Pyne S, Pyne NJ. (2006)The functional PDGFbeta receptor-S1P1 receptor signaling complex is involved in regulating migration of mouse embryonic fibroblasts in response to platelet derived growth factor. *Prostaglandins Other Lipid Mediat.* **80**,74-80.

- Lopez-Ilasaca M, Gutkind JS, Wetzker R. (1998) Phosphoinositide 3-kinase gamma is a mediator of Gbetagamma-dependent Jun kinase activation. *J Biol Chem*. **273**, 2505-2508.
- Ma AD, Metjian A, Bagrodia S, Taylor S, Abrams CS. (1998) Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase gamma, a Rac guanosine exchange factor, and Rac. *Mol Cell Biol*. **18**, 4744-4751.
- MacDougall LK, Domin J, Waterfield MD. (1995) A family of phosphoinositide 3-kinases in Drosophila identifies a new mediator of signal transduction. *Curr Biol.* **5**, 1404-1415.
- Maier U, Babich A, Nurnberg B. (1999) Roles of non-catalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma. *J Biol Chem*. **274**, 29311-29317.
- Malchinkhuu E, Sato K, Horiuchi Y, Mogi C, Ohwada S, Ishiuchi S, Saito N, Kurose H, Tomura H, Okajima F. (2005) Role of p38 mitogen-activated kinase and c-Jun terminal kinase in migration response to lysophosphatidic acid and sphingosine-1-phosphate in glioma cells. *Oncogene*. **24**, 6676-6688.
- Mehta D, Konstantoulaki M, Ahmmed GU, Malik AB. (2005) Sphingosine 1-phosphate-induced mobilization of intracellular Ca2+ mediates rac activation and adherens junction assembly in endothelial cells. *J Biol Chem.* **280**, 17320-17328.
- Metjian A, Roll RL, Ma AD, Abrams CS. (1999) Agonists cause nuclear translocation of phosphatidylinositol 3-kinase gamma. A Gbetagamma-dependent pathway that requires the p110gamma amino terminus. *J Biol Chem*. **274**, 27943-27947.
- Molz L, Chen YW, Hirano M, Williams LT. (1996) Cpk is a novel class of Drosophila PtdIns 3-kinase containing a C2 domain. *J Biol Chem*. **271**, 13892-13899.

- Morales-Ruiz M, Lee MJ, Zollner S, Gratton JP, Scotland R, Shiojima I, Walsh K, Hla T, Sessa WC. (2001) Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J Biol Chem*. **276**, 19672-19677.
- Murga C, Fukuhara S, Gutkind JS. (2000) A novel role for phosphatidylinositol 3-kinase beta in signaling from G protein-coupled receptors to Akt. *J Biol Chem*. **275**, 12069-12073.
- Murohara T, Witzenbichler B, Spyridopoulos I, Asahara T, Ding B, Sullivan A, Losordo DW, Isner JM. (1999) Role of endothelial nitric oxide synthase in endothelial cell migration. *Arterioscler Thromb Vasc Biol*. **19**, 1156-1161.
- Nobes CD, Hall A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*. **81**, 53-62.
- Nolte RT, Eck MJ, Schlessinger J, Shoelson SE, Harrison SC. (1996) Crystal structure of the PI 3-kinase p85 amino-terminal SH2 domain and its phosphopeptide complexes. *Nat Struct Biol*. **3**, 364-374.
- Ohmori T, Yatomi Y, Okamoto H, Miura Y, Rile G, Satoh K, Ozaki Y. 2001 G(i)-mediated Cas tyrosine phosphorylation in vascular endothelial cells stimulated with sphingosine 1-phosphate: possible involvement in cell motility enhancement in cooperation with Rho-mediated pathways. *J Biol Chem*. **276**, 5274-5280.
- Osawa Y, Banno Y, Nagaki M, Brenner DA, Naiki T, Nozawa Y, Nakashima S, Moriwaki H. (2001) TNF-alpha-induced sphingosine 1-phosphate inhibits apoptosis through a phosphatidylinositol 3-kinase/Akt pathway in human hepatocytes. *J Immunol*. **167**,173-180.
- Paik JH, Chae Ss, Lee MJ, Thangada S, Hla T. (2001) Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of alpha vbeta3- and beta1-containing integrins. *J Biol Chem.* **276**, 11830-11837.

- Panetti TS, Nowlen J, Mosher DF. 2000 Sphingosine-1-phosphate and lysophosphatidic acid stimulate endothelial cell migration. *Arterioscler Thromb Vasc Biol*. **20**, 1013-1019.
- Pestonjamasp KN, Forster C, Sun C, Gardiner EM, Bohl B, Weiner O, Bokoch GM, Glogauer M. (2006) Rac1 links leading edge and uropod events through Rho and myosin activation during chemotaxis. *Blood*. **108**, 2814-2820.
- Prasad KV, Janssen O, Kapeller R, Raab M, Cantley LC, Rudd CE. (1993) Src-homology 3 domain of protein kinase p59fyn mediates binding to phosphatidylinositol 3-kinase in T cells. *Proc Natl Acad Sci*. **90**, 7366-7370.
- Puri KD, Doggett TA, Huang CY, Douangpanya J, Hayflick JS, Turner M, enninger J, Diacovo TG. (2005) The role of endothelial PI3Kgamma activity in neutrophil trafficking. *Blood*. **106**, 150-157.
- Qian Y, Corum L, Meng Q, Blenis J, Zheng JZ, Shi X, Flynn DC, Jiang BH. (2004) PI3K induced actin filament remodeling through Akt and p70S6K1: implication of essential role in cell migration. *Am J physiol cell physiol*, **286**, 153-163.
- Rameh LE, Chen CS, Cantley LC. (1995) Phosphatidylinositol $_{(3,4,5)}$P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell*. **83**, 821-830.
- Rickert P, Weiner OD, Wang F, Bourne HR, Servant G. (2000) Leukocytes navigate by compass: roles of PI3Kgamma and its lipid products. *Trends Cell Biol*. **10**, 466-473.
- Ridley AJ, Hall A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. **70**, 389-399.
- Rikitake Y, Hirata K, Kawashima S, Ozaki M, Takahashi T, Ogawa W, Inoue N, Yokoyama M. (2002) Involvement of endothelial nitric oxide in sphingosine-1-phosphate-induced angiogenesis. *Arterioscler Thromb Vasc Biol*. **22**, 108-114.

- Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O, Takuwa Y. (2002) Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ Res*. **90**, 325-332.
- Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B., Wakeham A, Itie A, Bouchard D, Kozieradzki I, Joza N, Mak TW, Ohashi PS, Suzuki A, Penninger JM. (2000) Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science*, **287**, 1040- 1046.
- Schu PV, Takegawa K, Fry MJ, Stack JH, Waterfield MD, Emr SD. (1993) Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science*. **260**, 88-91.
- Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW, Bourne HR. (2000) Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science*. **287**, 1037-1040
- Singleton PA, Dudek SM, Chiang ET, Garcia JG. (2005) Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. *FASEB J.* **19**, 1646-1656.
- Skurk C, Maatz H, Rocnik E, Bialik A, Force T, Walsh K. (2005) Glycogen-Synthase Kinase3beta/beta-catenin axis promotes angiogenesis through activation of vascular endothelial growth factor signaling in endothelial cells. *Circ Res*. **96**, 308-318.
- Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, Hawkins PT. (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science*. **279**, 710-714.
- Stephens L, Jackson T, Hawkins PT. (1993) Synthesis of phosphatidylinositol 3,4,5-trisphosphate in permeabilized neutrophils regulated by receptors and G-proteins. *J Biol Chem.* **268**, 17162-17172.

- Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, Smrcka AS, Thelen M, Cadwallader K, Tempst P, Hawkins PT. (1997) The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell*. **89**, 105-114.
- Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science*. **277**, 567-570.
- Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, Stoyanova S, Vanhaesebroeck B, Dhand R, Nurnberg B. (1995) Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science.* **269**, 690-693.
- Suire S, Coadwell J, Ferguson GJ, Davidson K, Hawkins P, Stephens L. (2005) p84, a new Gbetagamma-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110gamma. *Curr Biol*. **15**, 566-570.
- Sutton RB, Davletov BA, Berghuis AM, Sudhof TC, Sprang, SR. (1995) Structure of the first C2 domain of synaptotagmin I: a novel Ca2+/phospholipid-binding fold. *Cell.* **80**, 929-938.
- Tang X and Downes CP. (1997) Purification and characterization of Gbetagamma-responsive phosphoinositide 3-kinases from pig platelet cytosol. *J Biol Chem*. **272**, 14193-14199.
- Thompson B, Ancellin N, Fernandez SM, Hla T, Sha'afi RI. 2006 Protein kinase Calpha and sphingosine 1-phosphate-dependent signaling in endothelial cell. *Prostaglandins Other Lipid Mediat*. **80**, 15-27.
- Tolias KF, Cantley LC, Carpenter CL. (1995) Rho family GTPases bind to phosphoinositide kinases. *J Biol Chem*. **270**, 17656-17659.
- Tu C, Koenning SR, Hu S. (2003) Root-Parasitic Nematodes Enhance Soil Microbial Activities and Nitrogen Mineralization. *Microb Ecol*. **46**, 134-144.

- Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci.* **22**, 267-272.
- Vanhaesebroeck B, Higashi K, Raven C, Welham M, Anderson S, Brennan P, Ward SG, Waterfield MD. (1999a) Autophosphorylation of p110delta phosphoinositide 3-kinase: a new paradigm for the regulation of lipid kinases in vitro and in vivo. *Embo J*. **18**, 1292-1302.
- Vanhaesebroeck B, Jones GE, Allen WE, Zicha D, Hooshmand-Rad R, Sawyer C, Wells C, Waterfield MD, Ridley AJ. (1999b) Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. *Nat Cell Biol*. **1**, 69-71.
- Vouret-Craviari V, Bourcier C, Boulter E, van Obberghen-Schilling E. (2002) Distinct signals via Rho GTPases and Src drive shape changes by thrombin and sphingosine-1-phosphate in endothelial cells. *J Cell Sc*i. **115**, 2475-2484.
- Vogel LB, Fujita DJ. (1993) The SH3 domain of p56lck is involved in binding to phosphatidylinositol 3'-kinase from T lymphocytes. *Mol Cell Biol.* **13**, 7408-7417.
- Voigt P, Dorner MB, Schaefer M. (2006) Characterization of p87PIKAP, a novel regulatory subunit of phosphoinositide 3-kinase gamma that is highly expressed in heart and interacts with PDE3B. *J Biol Chem*. **281**, 9977-9986.
- Walker EH, Perisic O, Ried C, Stephens L, Williams RL. (1999) Structural insights into phosphoinositide 3-kinase catalysis and signalling. *Nature*. **402**, 313-320.
- Wang F, Herzmark P, Weiner OD, Srinivasan S, Servant G, Bourne HR. (2002) Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat Cell Biol*. **4**, 513-518
- Wang F, Van Brocklyn JR, Hobson JP, Movafagh S, Zukowska-Grojec Z, Milstien S, Spiegel S. (1999a) Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis. *J Biol Chem*. **274**, 35343-35350.

- Wang F, Van Brocklyn JR, Edsall L, Nava VE, Spiegel S. (1999b) Sphingosine-1-phosphate inhibits motility of human breast cancer cells independently of cell surface receptors. *Cancer Res.* **59**, 6185-6191.
- Weiss-Haljiti C, Pasquali C, Ji H, Gillieron C, Chabert C, Curchod ML, Hirsch E, Ridley AJ, van Huijsduijnen RH, Camps M, Rommel C. (2004) Involvement of phosphoinositide 3-kinase gamma, Rac, and PAK signaling in chemokine-induced macrophage migration. *J Biol Chem*. **279**, 43273-43284.
- Wu D, Huang CK, Jiang H. (2000) Roles of phospholipid signaling in chemoattractant-induced responses. *J Cell Sci*. **113**, 2935-2940.
- Wyszomierski SL, Yu D. (2005) A knotty turnabout?: Akt1 as a metastasis suppressor. *Cancer Cell*. **8**, 437-439.
- Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. (2005) Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell*. **20**, 539-550.
- Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer, JM. (1998) Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol*. **18**, 1379-1387.
- Zhang B, Cao H, Rao GN. (2005) 15(S)-hydroxyeicosatetraenoic acid induces angiogenesis via activation of PI3K-Akt-mTOR-S6K1 signaling. *Cancer Res*. **65**, 7283-7291.
- Zheng Y, Bagrodia S, Cerione RA. (1994) Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. *J Biol Chem*. **269**, 18727-18730.
- Zhou GL, Tucker DF, Bae SS, Bhatheja K, Birnbaum MJ, Field J. (2006) Opposing roles for Akt1 and Akt2 in Rac/Pak signaling and cell migration. *J Biol Chem*. **281**, 36443-36453.

Zusammenfassung

Phosphoinositid-3-Kinasen (PI3-Kinasen) spielen in vielen zellulären Prozessen wie Proliferation, Inflammation und Migration eine zentrale Rolle. Die beiden Isoformen PI3-Kinase γ und β werden nach Stimulierung von G_i-Protein-gekoppelten Rezeptoren durch verschiedene Agonisten aktiviert und sind in die Regulation der Zellmotilität einbezogen. PI3-Kinase γ ist beispielsweise ein Schlüsselmediator bei der durch Chemokine stimulierten Migration von Leukozyten. PI3-Kinasen beeinflussen die Zellmigration vor allem über die Proteinkinase Akt und verschiedene Guanin-Nukleotid-Austausch-Faktoren (GEFs), die zur Aktivierung der kleinen GTPase Rac führen.

Sphingosin-1-phosphat (S1P), ein wirkungsvoller Angiogenesefaktor, aktiviert PI3-Kinasen, Akt und Rac in Endothelzellen über seinen an G_i-Proteine gekoppelten Rezeptor $S1P_1$ und reguliert auf diese Weise die Migration von Endothelzellen. Bisher ist kaum bekannt, welche PI3-Kinase-Isoformen durch S1P stimuliert werden und die S1P-induzierte Migration von Endothelzellen vermitteln. In der vorliegenden Arbeit wurde daher die Rolle der PI3-Kinase-Isoformen γ und β in der S1P-induzierten Signaltransduktion und Migration in humanen Nabelschnur-Endothelzellen (HUVEC) und in murinen mikrovaskulären Lungen-Endothelzellen (MLEC) untersucht.

Unsere Daten zeigen, dass die katalytischen Untereinheiten von PI3-Kinase γ und β in HUVEC und MLEC exprimiert sind. Im Gegensatz dazu konnte die mRNA der regulatorischen Untereinheiten der PI3-Kinase γ, p101 und $p87^{PIKAP}$, nur in MLEC, nicht aber in HUVEC nachgewiesen werden. Dieser Befund lässt vermuten, dass PI3-Kinase γ auch ohne diese Adapterproteine funktionsfähig ist.

Um die Rolle der beiden PI3-Kinase-Isoformen zu charakterisieren, wurde die Aktivität von PI3-Kinase β oder PI3-Kinase γ mit spezifischen Inhibitoren (TGX-221 für PI3-Kinase β, AS-252424 für PI3-Kinase γ) oder durch Überexpression der dominant-negativen Mutanten p110β-KR oder p110γ-KR inhibiert. Außerdem wurden Experimente in MLEC aus PI3-Kinase-γ-Knockout-Mäusen ($p110\gamma^{-/-}$) durchgeführt.

Unsere Ergebnisse bestätigen, dass S1P sowohl die Phosphorylierung von Akt und seines nachgeschalteten Effektors, der endothelialen NO-Synthase (eNOS), induziert als auch die Aktivierung von Rac stimuliert. Diese Prozesse waren PI3-Kinase-abhängig, da sie durch den PI3-Kinase-Inhibitor Wortmannin gehemmt wurden. Interessanterweise wurde die S1P-stimulierte Akt-Phosphorylierung vollständig und die eNOS-Phosphorylierung teilweise durch PI3-Kinase β vermittelt, während die Inhibition von PI3-Kinase γ keinen Einfluss auf den Akt/eNOS-Signalweg hatte. Im Gegensatz dazu wurde die durch S1P induzierte Rac-Aktivität sowohl durch Inhibition der PI3-Kinase β (TGX-221) als auch Inhibition der PI3-Kinase γ (AS-252424) verringert. Diese Daten zeigen daher, dass beide Isoformen an der durch S1P induzierten Rac-Aktivierung beteiligt sind. Dementsprechend war die Rac-Aktivierung durch S1P in p110$\gamma^{-/-}$-MLEC ebenfalls vermindert.

Zur Untersuchung der S1P-induzierten Migration wurden Transwell-Migrationsassays und Wound-Healing-Assays durchgeführt. Unsere Ergebnisse bestätigen, dass S1P die Migration von HUVEC und MLEC über G_i-Protein- und PI3-Kinase-abhängige Signalwege auslöst, da sie durch Pertussis-Toxin, einen $G_{i/o}$-Protein-Inhibitor, oder Wortmannin gehemmt wurden. Im Transwell-Assay, bei dem die Bewegung der Zellen in Richtung eines S1P-Gradienten untersucht wurde, führte sowohl die Hemmung der PI3-Kinase β als auch die der PI3-Kinase γ zu einer signifikanten Reduktion der Migration. Ein ähnliches Ergebnis hatte die Expression der dominant-negativen Mutanten beider Isoformen. Auch p110$\gamma^{-/-}$-MLEC wiesen eine verminderte Fähigkeit auf, sich entlang des S1P-Gradienten zu bewegen, und diese bereits reduzierte Reaktion wurde durch Inhibition der PI3-Kinase β noch weiter verringert. Im Gegensatz dazu scheint die Migration im Wound-Healing-Assay, die durch eine konstante Dosis von S1P induziert wird, nur von der Aktivierung der PI3-Kinase β abzuhängen. Unsere Daten zeigen, dass lediglich die Inhibition von PI3-Kinase β, aber nicht von PI3-Kinase γ, in der Lage war, diesen Prozess zu beeinträchtigen. Diese Ergebnisse lassen uns schlussfolgern, dass sowohl PI3-Kinase β als auch PI3-Kinase γ für die gerichtete Zellbewegung benötigt werden,

während die Migration ohne chemischen Gradienten nur PI3-Kinase β erfordert. Eine mögliche Funktion der PI3-Kinase γ könnte darin bestehen, die Zellfront („leading edge“) durch lokale Produktion von $PI_{(3,4,5)}P_3$ zu stabilisieren. Wie bereits in Neutrophilen beschrieben wurde, könnte das zu einer lokalen Rekrutierung von Proteinen mit PH-Domäne und Polymerisation von F-Aktin führen.

Wir vermuten, dass die beiden untersuchten PI3-Kinase-Isoformen unterschiedliche Signalwege steuern, wobei Akt der Haupteffektor von PI3-Kinase β ist und Rac einen nachgeschalteten Mediator beider PI3-Kinasen darstellt. Möglicherweise werden unterschiedliche Rac-GEF-Proteine von PI3-Kinase β und γ aktiviert. Unsere Ergebnisse gewähren neue Einblicke in die Regulation der Migration von Endothelzellen, welche einen essentiellen Prozess der Angiogenese darstellt.

Summary

Phosphoinositide 3-kinases (PI3Ks) have been indicated to play a central role in many cellular processes, such as proliferation, inflammation and migration. Two isoforms, PI3Kβ and PI3Kγ, are known to be activated by G_i proteins in response to different stimuli and to regulate cell motility. For instance, PI3Kγ has been demonstrated to be a key mediator in chemoattractant-stimulated leukocyte migration. Downstream mediators of PI3Ks involved in cell migration include the protein kinase Akt and guanine nucleotide exchange factors (GEFs) which lead to the activation of the small GTPase Rac.

Sphingosine 1-phosphate (S1P), a potent angiogenic factor, is known to activate PI3Ks , Akt and Rac in endothelial cells via its G_i protein-coupled receptor $S1P_1$ and to regulate endothelial cell migration. However, so far the function of PI3Kβ and PI3Kγ isoforms in S1P-induced endothelial migration is poorly understood. In the present study, we investigated the role of both PI3K isoforms in S1P-induced signalling and migration in human umbilical vein endothelial cells (HUVEC) and in mouse lung microvascular endothelial cells (MLEC).

Our data show that the catalytic subunits of both, PI3Kβ and PI3Kγ, are expressed in HUVEC and MLEC at the protein level. In contrast, mRNA of the regulatory subunits of PI3Kγ, p101 and $p87^{PIKAP}$, was only detected in MLEC but not in HUVEC suggesting that PI3Kγ can be functional in the absence of these adaptor proteins.

To characterize the role of PI3K isoforms, we selectively targeted PI3Kβ or PI3Kγ activities by pretreating endothelial cells with specific inhibitors (TGX-221 against PI3Kβ; AS-252424 against PI3Kγ), or by overexpressing dominant negative mutants of PI3Kβ (p110β KR) or PI3Kγ (p110γ KR). Furthermore, we performed studies in MLEC derived from PI3Kγ gene knockout ($p110\gamma^{-/-}$) mice.

Our data confirm that S1P stimulates the phosphorylation of Akt and its downstream effector endothelial nitric oxide synthase (eNOS) as well as Rac activation in a PI3K-dependent way since all parameters were inhibited by wortmannin, a general

PI3K inhibitor. Interestingly, only PI3Kβ was responsible for Akt phosphorylation and, partially, for eNOS phosphorylation, whereas PI3Kγ inhibition had no effect on the Akt/eNOS pathway. In contrast, inhibition of PI3Kβ or PI3Kγ by TGX-221 or AS-252424, respectively, attenuated Rac activity in response to S1P stimulation indicating that both isoforms are involved in S1P-induced Rac activation. Accordingly, Rac activation in response to S1P was diminished in $p110\gamma^{-/-}$ MLEC.

To investigate S1P-induced migration, a transwell migration assay and a wound healing assay were performed. Our data confirm that S1P induces HUVEC and MLEC migration through G_i protein- and PI3K-dependent pathways, since pertussis toxin, a $G_{i/o}$ protein inhibitor, and wortmannin revealed inhibitory effects. In the transwell assay, which investigates cell migration towards an S1P gradient, inhibition of both, PI3Kβ and PI3Kγ, resulted in a significant reduction of the migratory response. A similar result was observed when dominant negative mutants of both isoforms were exploited to inhibit enzyme activities. Moreover, $p110\gamma^{-/-}$ MLEC displayed less capability to move along an S1P gradient which was further reduced by inhibiting PI3Kβ in these cells. In contrast, migration in wound healing assays which was induced by a constant dose of S1P seems to be only dependent on PI3Kβ activation, since only specific targeting of PI3Kβ but not of PI3Kγ was able to impair this process. Based on these results, we suggest that both, PI3Kβ and PI3Kγ, are required for directional cell movement, whereas migration in the absence of a chemotactic gradient requires only PI3Kβ. A possible role of PI3Kγ may be to stabilise the leading edge of the cells by local production of $PI_{(3,4,5)}P_3$, which leads to neighboring recruitment of PH domain containing proteins and F-actin polymerisation as described in neutrophils.

We suggest that the two PI3K isoforms signal through different pathways with Akt being a major effector of PI3Kβ and Rac being a downstream mediator of both PI3Ks. We also suggest that different Rac-GEFs may be activated by PI3Kβ or PI3Kγ . Our results provide new insights in the regulation of endothelial migration which is an essential process in angiogenesis.

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Publikationen in Monographien

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PI3Kβ and PI3Kγ control motility of endothelial cells induced by sphingosine 1-phosphate.

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The phosphatidylinositol 3-kinase β isoform regulates sphingosine 1-phosphate-induced Akt-activation and motility in endothelial cells

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Jena, den 8.Jan 2007

Erklärung zur Bewerbung

Hiermit erkläre ich, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad doctor rerum naturalium beworben habe, und dass weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des oben genannten akademischen Grades an einer anderen Hochschule beantragt habe.

Jena, den 8.Jan 2007